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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Brakel et al.)	Group Art Unit: 1631
)	
Serial No.:	08/479, 999)	Examiner: Ardin Marschel
)	
Filed:	June 28, 1994)	
)	
For:	MODIFIED NUCLEOTIDE COMPOUNDS)	
)	

527 Madison Avenue (9th Floor)
New York, New York 10022
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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

I. REAL PARTY IN INTEREST

The real party in interest of the present application is Enzo Therapeutics, Inc., which is a subsidiary of Enzo Biochem., Inc. (hereinafter "enzo"). Enzo is the owner of the present application by way of an assignment from the inventors, Brakel et al., of all rights, title, and interest.

II. RELATED APPEALS AND INTERFERENCES

There are no appeals or interferences related to the present application.

III. STATUS OF CLAIMS

All of the pending claims are rejected. A copy of the pending claims is attached hereto in the Appendix.

IV. STATUS OF AMENDMENTS

After the Final Office Action dated September 26, 2000, the following were submitted by Appellants: an amendment under 37 C.F.R. 1.116 in response to the Office Action dated September 26, 2000. This submission does not appear to have been acted upon by the Office.

V. SUMMARY OF INVENTION

The present invention is directed to modified nucleotide compounds complementary to at least a portion of and effective to inhibit the function of an RNA of an organism when administered. These compounds are resistant to nucleases yet form an RNase H substrate when hybridized to a complementary RNA sequence. The modified compound comprises at least one component selected from the group consisting of MN_3 , M , $B(N)_x$, M and $M(N)_x$, B wherein: N is a phosphodiester-linked modified or unmodified 2'-deoxynucleoside moiety, preferably unmodified; M is a moiety that confers endonuclease resistance on said component and that contains at least one modified or unmodified nucleic acid base; B is a moiety that confers exonuclease resistance to the terminus to which it is attached; x is an integer of at least 2, preferably at least two or three (see specification, page 5, lines 8-18 and original claim 1). M and B may be the same or different. Furthermore, M and B may

confer both endonuclease and exonuclease resistance (see specification, page 7, lines 28-29).

In one specific embodiment, N may be modified or unmodified. Modified forms include but are not limited to 2,6-diaminopurine, uracil, inosine, 5-halogenated uracil or cytosine, substituted or unsubstituted 7-deazaguanine, 7-deazaadenine, 7-deazainosine, or a methylated adenine, thymine, cytosine or guanine (see specification, page 7, lines 15-20 and original claims 4-7).

In another specific embodiment, M may be a C₁-C₄ alkylphosphonate, such as methylphosphonate or an alpha-phosphodiester linkage. Other examples include but are not limited to an aminophosphonate, phosphotriester, phosphoramidate, carbamate or morpholino-substituted nucleotide (see specification, page 7, lines 19-28, original claims 8-11).

B may be attached directly or indirectly to the deoxyribose moiety of at least one of the 3' and 5' terminal nucleotides. Examples of B include an intercalating agent, a methylthiophosphate, a carbodiimide and an N-hydroxybenzotriazole, an isourea, a polypeptide or protein or modified or unmodified 2',3'-deoxyribose nucleotide (see paragraph bridging pages 7 and 8 and original claims 12-18).

The invention is further directed to methods of using the compounds of the present invention. Specifically, said compounds may be used to inhibit the function of an RNA (claim 21) and/or treating a human or mammal so as to inhibit the function of its target RNA (claim 41).

The invention is also directed to a method for identifying oligo- or polynucleotides having a combination of nuclease resistance and the ability to form an RNase H substrate when in an RNA:oligo- or polynucleotide complex (see paragraph bridging pages 8 and 9 and claim 40).

VI. ISSUES

There are four issues on appeal. First, whether claims 1-19, 21-39, 41, 51, and 52 are unpatentable under 35 U.S.C. 112, first paragraph as lacking adequate written description. Second, whether claims 1, 2, 4, 8, 12-14, 19 and 42-50 are unpatentable over 35 U.S.C. §102(b) over Miller et al., 1985, *Biochimie* 67:769-776 (hereinafter "Miller et al., 1985"). Third, whether claims 1-4, 12-14 and 42-50 are unpatentable under 35 U.S.C. 102(b) over Stein et al., 1988, *Nucl. Acids Res.* 16:3209-3221 (hereinafter "Stein et al."). Fourth whether claims 1-52 are unpatentable under 35 U.S.C. §103(a) over Walder et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:5011-5015 (hereinafter "Walder et al.") in view of Ts'o et al., 1984, U.S. Pat. No. 4,469,863 (hereinafter "Ts'o et al.") and Inoue et al., 1987, *Nucl. Acids Res. Symposium Series No. 18*, pp. 221-224 (hereinafter "Inoue et al.>").

VII. GROUPING OF CLAIMS

Appellants submit that claims 1-52 do not stand or fall together. Each of claims 5-11, 15-20, 24-30, 34-40 and 42-52 are drawn to specific embodiments that include an element that is not shown or suggested in the cited art. Therefore, Appellants submit that each of the following groups of claims separately stand and fall together:

- (1) Claims 1-4, 12-14, 21-23, 31-33, 41
- (2) Claims 5-7, 10-11, 15-19, 24-26, 29-30, 34-38
- (3) Claims 8-9, 20, 27-28 and 39 stand and fall together
- (4) Claim 40 stands and falls together

- (5) Claims 42-50 stand and fall together
- (6) Claim 51 stands and falls together
- (7) Claim 52 stands and falls together

VIII. ARGUMENT

The present invention is directed to novel modified nucleotide compounds that are contain endo and exonuclease resistant components and can form RNase H substrates when complexed with a complementary RNA as well as a method for identifying such compounds. The invention is also directed to methods of using these compounds, specifically, inhibiting the function of an RNA (claim 21) and treating a human or animal so as to inhibit the function of a target RNA as well as methods for identifying such compounds. The subject matter recited in the pending claims is adequately described so that one of skill in the art would undoubtedly appreciate from this disclosure that the inventors were in possession of all of the claimed subject matter at the time of filing. Furthermore, the claimed subject matter is not anticipated by Miller et al., 1985, or Stein et al. Finally, the claimed subject matter is not obvious over Walder et al., in view of Ts'o et al. and Inoue et al.

A. The Claims as Amended do not contain New Matter

1. Summary of the Prosecution History

Appellants, in response to the Office Action dated January 4, 1999, amended claims 1, 21, 41 and 51 to recite that the modified nucleotide compound includes at least one component selected from the group consisting of MN_3M , $(N)_xM(N)_y$, $(N)_xM(N)_yM$, $B(N)_xM(N)_y$ and $(N)_xM(N)_yB$, where N is a phosphodiester-linked modified or unmodified 2'-deoxynucleoside moiety; provided that at least one N is a

phosphodiester-lined unmodified 2' deoxynucleoside moiety; M is a moiety that confers endonuclease resistance on said component and that contains at least one modified or unmodified nucleic acid base; B is a moiety that confers exonuclease resistance. In the Final Office Action dated September 26, 2000, it is asserted that these claim amendments constitute new matter.

2. No new matter has been added

35 U.S.C. §112, first paragraph, requires that an applicant provide “a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise and exact terms...” to fulfill the written description requirement, a patent specification must describe the invention and do so in sufficient detail that one skilled in the art can clearly conclude that the inventor possessed the claimed invention at the time of filing. *Lockwood v. American Airlines, Inc.* 107 F.3d 1565 (Fed. Cir. 1997); see also *Regents of the Univ. of Cal. V. Eli Lilly & Co.*, 119 F.3d 1559 (Fed. Cir. 1997). Satisfaction of the written description requirement does not require *in haec verba* antecedence in the originally filed application. *Staehelin v. Secher*, 24 USPQ2d 1513 (Bd. Pat. App. & Interfer. 1992).

As noted in MPEP §2163.01

A written description requirement issue generally involves the question of whether the subject matter of a claim is supported by [conforms to] the disclosure of an application as filed. If the examiner concludes that the claimed subject matter is not supported [described] in an application as filed, this would result in a rejection of the claim on the ground of a lack of written description under 35 U.S.C. 112, first paragraph or denial of the benefit of the filing date of a previously filed application.

The position asserted in the MPEP is summarized in a Decision Tree (see **Tab 1**) included in Training Materials for Examiners on Written Description, available from <http://www.uspto.gov/web/patents/guides.htm>.

It is Appellant's position that there is actually support in the specification for MN_3M , $(N)_xM(N)_y$, $(N)_xM(N)_yM$, $B(N)_xM(N)_y$ and $(N)_xM(N)_yB$. MN_3M was of course recited in the original claims. Examples of $(N)_xM(N)_y$, $(N)_xM(N)_yM$, $B(N)_xM(N)_y$ and $(N)_xM(N)_yB$ are provided in the specification and include TGACTTAGCTGCAT, TGACTTAGCTGCAT, TGACTTAGCTGCAT, TGACTTAGCTGCAT, TGACTTAGCTGCAT, TGGGGCTGCATG, CTGGGGCTGCAT (see Table 1, page 11 and page 19).

It is well established case law that where there is no explicit description of a generic invention in the specification, mention of representative compounds may provide an implicit description upon which to base generic claim language. *In re Smith*, 458 F.2d 1389, 173 USPQ 679 (CCPA 1972) and *Ralston Purina v. Far-Mar-Co., Inc.*, 228 USPQ 863 (Fed. Cir. 1985). Specifically in *Smith*, disclosure in the parent application of the following polar organic compounds that involve surface coating the pigment with an oil soluble polar organic compound: acidic resins, water soluble resonates, water insoluble metallic resonates, long chain fatty acids, their salts and soaps, benzene carboxylic acid and its slats, naphthenic acids and their soaps and salts, cationic active agents, e.g., alkyl amine salts and quaternary ammonium compounds containing at least 12 carbon atoms in an alkyl group or groups, e.g., lauryl pyridinium bromide, and long chain (at least 12 carbon atoms) fatty acid-containing organic Werner complexes is considered to provide adequate support in a subsequent application of a claim to an emulsion coating composition comprising an organic material used to coat pigment as being monomeric and

containing at least 8 carbon atoms and at least one carboxy or carboxylate group.

The court in *Smith* stated

It is obvious that the surface coating organic compounds recited in the foregoing paragraph are monomeric, have a hydrocarbon structure of at least 8 carbon atoms, except for benzene carboxylic acid which contains six carbon atoms in a hydrocarbon group, and contain at least one carboxy or carboxylate group. If appellant's claims had been drawn more broadly, they would be supported by the parent application. They can be described as subgeneric claims because they delineate the invention more specifically by reciting that the organic material used to coat the pigment is monomeric, contains at least 8 carbon atoms and at least one carboxy or carboxylate group.

In *Ralston Purina*, the court held that the disclosures "soybean meal having a low fat and high protein content", "such 50% protein soybean meal is well known and frequently is a by-product of the process of oil extraction from soybeans. Such meal is preferably solvent extracted to decrease the fat content thereof to the range mentioned above" and "soybean meal having a protein content of approximately 50% is the preferred meal component for use in the present invention. When however, the meal has a protein content of substantially less than 50%, it may be mixed with a high protein component which will increase the protein content of the combination to the preferred 50%" in an earlier filed application supports in a subsequently filed application the claim language "protein content of at least about that of solvent extracted soybean meal".

It is respectfully submitted that the amendments to claims 1, 21, 41 and 51 do not constitute new matter since there is adequate support for said claims.

Furthermore, Applicants submit herewith is a Second Amendment under 37 C.F.R. 1.116 in accordance with MPEP §1207. This amendment contains new claims 53-54 and are supported by the specification. It is believed that these claims contain allowable subject matter.

A. The Claimed invention Is not Anticipated by the Cited Prior Art

1. Claims 1, 2, 4, 8, 12-14, 19 and 42-50 are not anticipated by Miller et al.

The originally pending claims 1, 2, 4, 8, 12-14, 19 and 42-50 were rejected under 35 U.S.C. §102(b) as being anticipated by Miller et al., 1985. In response to the Office Action dated March 31, 1998, Applicants amended claims 1 and 21 to recite

N is a phosphodiester-linked modified or unmodified 2'-deoxynucleoside moiety; provided that at least one N is a phosphodiester-linked unmodified 2' deoxynucleoside moiety

and amended claims 42 and 50 to add "wherein at least one of said contiguous phosphodiester-linked 2'-deoxynucleosides is unmodified".

The rejection was maintained and the Office Action dated January 4, 1999 stated:

Applicants argue that Miller et al. discloses oligodeoxyribonucleotides which are ALL modified with methylphosphonates. In response, Miller et al. on page 772, Figure 3, discloses the oligomer given as: 32pTpGpCpApCpCpApT. The notation "p" denotes a normal phosphodiester-linkage. Thus, at least one such

linkage is present in this oligomer as required in the newly submitted limitation of instant claim 1, for example, contrary to the allegation of applicants.

In the response filed on November 19, 1999, Appellants amended claim 1¹ to recite that the modified nucleotide compound which includes at least one component selected from the group consisting of MN_3M , $(N)_xM(N)_y$, $(N)_xM(N)_yM$, $B(N)_xM(N)_y$ and $(N)_xM(N)_yB$. MN_3M . The rejection was maintained in the final Office Action dated September 26, 2000. The Office Action specifically states;

Applicants argue that Miller et al. neither discloses nor suggests not fully modifying all of the internal phosphodiester linkages and is silent on RNase H sensitivity. This RNase H resistance argument is confusing in that instant claim 1, for example, lacks any mention of RNase H resistance or not. Only claims 44 and 49 as rejected hereinunder cite any RNase H practice and these claims, such as specifically claim 44 directs RNase H resistance to what is complexed with the compound of claim 42 and not the compound itself.

Appellants point out that anticipation requires that the claimed invention to have been known in the prior art "in the detail of the claim" such that each element and limitation contained in the claim is present in a single prior art reference, "arranged as in the claim". *Karsten Mfg. Corp. v. Cleveland Golf CO.* 242 F.3d 1376, 58 USPQ2d 1286 (Fed. Cir. 2001). Clearly, Miller et al., 1985 does not contain each and every element of the subject matter claimed.

To address this rejection in complete detail, Appellants will analyze each of the components of claims 1, 42 and 50. Appellants, with respect to claim 1, take issue

¹ Claims 21, 41 and 51 were also amended

with the assertion made in the Office Action that a methylphosphonate would be encompassed by the definition of "N". This is because while "M" and "B" are stated to confer endonuclease and exonuclease resistance on the claimed component, "N" is silent in that respect. Therefore it may be concluded that N would not confer nuclease resistance. If such an assumption is made, MN_3M , $(N)_xM(N)_y$, $(N)_xM(N)_yM$, $B(N)_xM(N)_y$ and $(N)_xM(N)_yB$ were not disclosed by Miller for the following reasons: in the case of MN_3M and $B(N)_xM(N)_y$ the sequence in Figure 3, begins with an ordinary unmodified 2'-deoxynucleoside moiety; in the case of $(N)_xM(N)_y$, and $(N)_xM(N)_yB$, the 5' terminus only contains one N. However, in order to put claims in condition for allowance or at least obviate some issues for appeal, submitted herewith is a Second Amendment under 37 C.F.R. §1.116 in accordance with MPEP §1207 where it is proposed that claims 1 and 21 be amended to recite that the compound when complexed with a complementary RNA acts as an RNase H substrate. It would thus follow that dependent claims 2, 4, 8, 12-14 and 19 would also not be anticipated by Miller et al., 1985.

With respect to claim 42, Appellants note that the sequences disclosed in Miller et al., 1985 either completely contain methylphosphonate linkages or just a 5'-phosphodiester unmodified moiety (see Figure 3 of Miller et al., 1985). None of the compounds disclosed in Miller et al., 1985 contain **two separate** nuclease resistant components. Additionally, Appellant in response to the comment made during prosecution, "Only claims 44 and 49 as rejected hereinunder cite any RNase H practice and these claims, such as specifically claim 44 directs RNase H resistance to what is complexed with the compound of claim 42 and not the compound itself", respectfully point out that RNase H sensitivity is only conferred when DNA and RNA complex together. It would thus follow that dependent claims 43-49 would also not

be anticipated by Miller et al., 1985. Furthermore, in order to put claim 42 in condition for allowance or at least obviate some issues for appeal, Appellants have submitted a Second Amendment under 37 C.F.R. §1.116 in accordance with MPEP §1207 where it is proposed that claim 42 be amended to that the claimed compound acts as an RNase H substrate when complexed with complementary RNA and that each nuclease resistant component comprises at least one moiety which confers endonuclease resistance and at least one moiety which confers exonuclease resistance and that 2 or more contiguous phosphodiester-linked 2' deoxynucleosides are located between the moiety conferring endonuclease resistance and the moiety conferring exonuclease resistance.

Appellants note that the cited Miller et al. reference is silent with respect to the RNase H sensitivity of the methylphosphonate sequences disclosed in Figure 3. However, it is most likely that these sequences are RNase H resistant. For example, Cazenave et al., 1989, Nucl. Acids Res. 17:4255-4273 (**Tab 2** and cited in the instant application) discloses that a methyl phosphonate 17-mer "failed to induce the degradation of the target mRNA by the E. coli RNase H". Additionally, Furdon et al., 1989, Nucl. Acids Res. 17:9193-9204 (**Tab 3**) discloses results from studies with a 14-mer oligonucleotide containing one to six methylphosphonate linkages. Results from the Furdon et al. studies indicated that "Susceptibility to cleavage by RNase H increased parallel to a reduction in the number of methylphosphonate residues in the oligonucleotide"². It is further noted in Furdon et al., 1989, Nucl. Acids Res. 17:9193-9204³

RNA hybridized to MP-oligos containing one or two
methylphosphonate deoxynucleosides was cleaved by

² See abstract in Furdon et al., 1989, Nucl. Acids Res. 17:9193-9204

³ See Furdon et al. at page 9202

RNase H almost as easily as that in the control duplex with D-oligo,,,RNA in duplexes with MP-oligos which contained three, four and six methylphosphonate deoxynucleosides, i.e., in which methylphosphonate bonds were separated by three, two or one phosphodiester bond...was increasingly resistant to cleavage by the enzyme.

Appellants also assert that claim 50 would also not be anticipated by Miller et al., 1985. Claim 50 recites that the compound must contain **two separate** nuclease resistant sequences. The compounds disclosed in Miller et al., 1985 do not contain two separate nuclease resistant sequences. Furthermore, in order to put claim 50 in condition for allowance or at least obviate some issues for appeal, Appellants have submitted a Second Amendment under 37 C.F.R. §1.116 in accordance with MPEP §1207 where it is proposed that claim 50 be amended to that the claimed compound acts as an RNase H substrate when complexed with complementary RNA and to more clearly recite that **each** nuclease resistant sequences each consists of 2 or 3 contiguous phosphodiester-linked 2'-deoxynucleosides.

It is Appellants' position that the properly interpreted rejected claims are not anticipated by Miller et al., 1985.

2. Claims 1-4, 12-14, and 42-50 are not anticipated by Stein et al., 1988, Nucl. Acids Res. 16:3209-3221

The originally pending claims were rejected under 35 U.S.C. §102(b) as being anticipated by Stein et al. As noted above, in response to the Office Action dated March 31, 1998, Applicants amended claim 1 to recite

N is a phosphodiester-linked modified or unmodified 2'-deoxynucleoside moiety; provided that at least one N is a

phosphodiester-linked unmodified 2' deoxynucleoside moiety.

and amended claims 42 and 50 to add "wherein at least one of said contiguous phosphodiester-linked 2'-deoxynucleosides is unmodified".

The rejection was maintained and the Office Action dated January 4, 1999 stated:

Applicants argue that Stein et al. motivates and suggest that ALL PS oligomers are the most desirable for increased RNase-H activity is desired and not to use S-capped oligomers or other non-fully modified oligomers. These arguments are non-persuasive in overcoming the rejection because the reference does disclose S-capped oligomers as well as non-fully modified oligomers and therefore reads on the instant claims, even if the reference also motivates and suggests certain preferred oligomer types.

In the response filed on November 19, 1999, Appellants amended claims 1, 21 and 51 to recite that the modified nucleotide compound which includes at least one component selected from the group consisting of MN_3M , $(N)_xM(N)_y$, $(N)_xM(N)_yM$, $B(N)_xM(N)_y$ and $(N)_xM(N)_yB$. The rejection was maintained in the final Office Action dated September 26, 2000. The Office Action specifically states;

Applicants argue again as above regarding RNase H resistance. This argument has been responded to above and is equally applicable here and is reiterated here. Applicants then argue regarding partial internal modification that is not in Stein et al. this is non-persuasive as it is based on the NEW MATTER added to the instant claims. This rejection is reiterated in anticipated of removal of the NEW MATTER thus leaving the claims rejected as before.

As with Miller et al., 1985, Stein et al. does not contain each and every element of the subject matter recited in the rejected claims. To address this rejection in complete detail, Appellants will analyze each of the components of claims 1, 42 and 50.

Again, as argued in response to the Written Description rejection, the amendments of claim 1 do not constitute new matter, since these claim amendments are actually supported by the specification. Appellants note that none of the above components recited in claim 1 are contained within the sequences disclosed. Specifically, Stein et al. discloses sequences containing all phosphorothioate linkages and a myc-1 oligomer sequence, 5'-AsAsCGTTGAGGGGCsAsT-3' ("s" depicts phosphorothioate linkages) containing two PS bonds at each end of the 15-mer. In the case of MN_3M , there are more than 3 "N" sequences between the two "Ms"; in the case of $(N)_xM(N)_yM$ and $(N)_xM(N)_yB$, none of the sequences in Stein et al., 1988 contain an "N" moiety at the 5' end; in the case of $B(N)_xM(N)_y$, the myc-1 sequence contains phosphorothioates at both the 5' and 3' ends.

With respect to claim 42, Appellants note that the sequences disclosed in Stein et al., either completely contain phosphorothioate linkages or as with the myc-1 oligomer, capped with phosphorothioates. None of the compounds disclosed in Stein et al., contain **two separate** nuclease resistant components. It would thus follow that dependent claims 43-49 would also not be anticipated by Stein et al. Furthermore, in order to put claim 42 in condition for allowance or at least obviate some issues for appeal, Appellants have submitted a Second Amendment under 37 C.F.R. §1.116 in accordance with MPEP §1207 where it is proposed that claim 42 be amended to that the claimed compound acts as an RNase H substrate when complexed with complementary RNA and that each nuclease resistant component

comprises at least one moiety which confers endonuclease resistance and at least one moiety which confers exonuclease resistance and that 2 or more contiguous phosphodiester-linked 2' deoxynucleosides are located between the moiety conferring endonuclease resistance and the moiety conferring exonuclease resistance.

Appellants also assert that claim 50 would also not be anticipated by Stein et al.. Claim 50 recites that the compound must contain **two separate** nuclease resistant sequences and consists of 2 or 3 contiguous phosphodiester-linked 2'-deoxynucleosides; wherein at least one of said contiguous phosphodiester-linked 2'-deoxynucleosides is unmodified. The compounds disclosed in Stein et al. do not contain two separate resistant sequences and certainly contain more than 2 or 3 contiguous phosphodiester-linked 2'-deoxynucleosides between the phosphorothioates at the 5' and 3' ends. Furthermore, in order to put claim 50 in condition for allowance or at least obviate some issues for appeal, Appellants have submitted a Second Amendment under 37 C.F.R. §1.116 in accordance with MPEP §1207 where it is proposed that claim 50 be amended to clearly recite that **each** nuclease resistant sequences each consists of 2 or 3 contiguous phosphodiester-linked 2'-deoxynucleosides.

It is Appellant's position that the properly interpreted rejected claims are not anticipated by Stein et al.

B. The Claimed invention Is not Obvious Over the Cited Prior Art

1. Recent Prosecution History

In the Office Action dated March 31, 1998, the Examiner referred to the rejection given in the Office Action mailed June 5 1992 which stated:

Enz-47(C)(2)

Walder et al. discloses that the most important element in the efficacy of antisense oligomers inhibiting mRNA expression is the formation of a RNase sensitive RNA-DNA duplex that is cleaved by the enzyme: "An important corollary of our results is that such modified analogs must not only retain normal hybridization properties but should also form substrates that are recognized and cleaved by RNase H"

Miller et al. ⁴discloses antisense oligomers with all methylphosphonate internucleotide linkages. These modified oligomers possess resistance to nucleases, can pass through the membranes of mammalian cells and can form stable duplexes with complementary mRNA ...

Inoue et al. teaches that a span as small as three contiguous phosphodiester linkages flanked by modified nucleotides (2'-O-methyl) was capable of forming an RNase H-sensitive substrate

The claimed modified oligonucleotides possess three primary characteristics: 1) endo- and exonuclease resistance, 2) ability to hybridize to its RNA complementary sequence, and 3) the ability to form a RNase sensitive RNA-DNA duplex.

The person of ordinary skill in the art with the above references before him would have found the claimed modified oligomers obvious because of the necessity to have reduced the number of methylphosphonate internucleotide bonds in the oligomer in order to make the RNA-DNA duplex RNase sensitive as Walder et al. emphasizes is critical to the efficacy of antisense oligonucleotides in inhibiting the express of mRNA.

The claimed methods of inhibiting the function of an RNA by contacting said RNA with a nuclease resistant antisense oligomer that forms RNase H sensitive

⁴ Miller et al. in the obviousness rejection is actually Ts'o et al.

duplexes with said RNA would also have been obvious in view of the above references that, as a whole, teach the same method.

Finally, the method for identifying modified antisense oligomers possessing the combination of nuclease resistance and the ability to form an RNase H substrate with complexes of RNA using gel electrophoresis instead of the release of acid soluble radioactivity as taught by Walder et al.... would have been obvious to the person of ordinary skill in the art. The use of gel electrophoresis is a fundamental tool in molecular biology for separating different types of polynucleotides whether by size or by other physical properties such as single-stranded versus double-stranded forms, linear versus circular forms, etc.

The applicant's basic invention is the antisense oligomer with only a portion of the internucleotide linkages or bases modified in order to make the oligomer nuclease resistant. However, the prior art clearly teaches the necessity of combining both nuclease resistance with the ability to form RNase H sensitive duplexes with RNA. The applicant's gel assay is only one way to assay for RNase H sensitivity as Walder et al. substantiates.

Appellants in their response filed on September 28, 1998 stated

Walder states that an important element for an effective antisense oligomer is that it be recognized and cleaved by RNase-H. However, Walder does not disclose or suggest that modifying a nucleotide compound, with sufficient spacing between modifications, might yield RNase-H sensitivity.

Miller (U.S. Patent No. 4,469,863) does not cure the deficiency of Walder. Miller is directed to fully modified oligomers and provides no disclosure or suggestion to only partially modify its oligomers, let alone that such would be RNase-H sensitive.

Inoue is concerned with a oligoribodeoxynucleotide probe comprised of modified RNA sequence attached to a DNA sequence attached to another modified RNA sequence. The modifications to the RNA sequences is to resist RNase-H—quite the opposite effect desired by Walder. Further, there is no suggestion or motivation provided by Inoue to modify its probe for use in antisense.

Accordingly, it is respectfully submitted that none of Walder, Miller or Inoue disclose or suggest Applicants' invention; that there is no suggestion or motivation to combine the disclosures of Walder, Miller and/or Inoue; and that, even if combined, the references do not disclose, suggest or otherwise render unpatentable Applicants' claimed invention.

In the Office Action dated January 4, 1999, the Examiner stated:

Applicants argue that Walder et al. states that antisense oligomers should be recognized and cleaved by RNase-H. This characterization is incorrect in that mRNA is cleaved by RNase-H after hybridization with the antisense oligomer. The antisense oligomer is not cleaved by RNase-H. Applicants then argue that Miller et al. is directed to only fully modified oligomers. This has been noted above as incorrect also because Miller et al. In Figure 3 therein discloses a partially modified oligomer and is not limited to fully linkage modified oligomers. Applicants then argue that Inoue et al. is directed to RNase-H resistance and not recognition by RNase-H. Consideration of Inoue et al. reveals that it is directed to the practice of antisense oligomers that mediate RNase-H cleavage of mRNA via modified portions, not necessarily all linkages therein, that are stable during antisense usage and still hybridize to mRNA. This reference was utilized in the rejection to support oligomer embodiments with modifications at the 2'-deoxyribose site and not regarding RNase-H resistance or sensitivity per se.

Appellants, as noted above, in their response submitted November 19, 1999, amended claims 1, 21, 41 and 51 to recite that the modified nucleotide compound which includes at least one component selected from the group consisting of MN_3M , $(N)_xM(N)_y$, $(N)_xM(N)_yM$, $B(N)_xM(N)_y$ and $(N)_xM(N)B$. Furthermore, Appellants stated

Walder states that an important element for an effective antisense oligomer is that it be recognized and cleaved by RNase-H. However, Walder does not disclose or suggest any means of modifying a nucleotide compound to decrease degradation while retaining RNase H-sensitivity. It most certainly does not suggest that modifying some of the internal nucleotides with sufficient spacing between modifications, might yield both nuclease resistance and RNase-H sensitivity.

Miller (U.S. Patent No. 4,469,863) does not cure the deficiency of Walder. Miller is directed to fully modified oligomers and provides no disclosure or suggestion to only partially modify its oligomers, let alone that such would be RNase-H sensitive. (The Examiner's reference to Figure 3 of the Miller patent seems to be a misstatement and that the Examiner is actually referring to Figure 3 of the Miller article. As discussed above, the Miller article does not cure the defects of Walder).

Inoue also does not cure the defects of Walder or Miller. Inoue is concerned with a oligoribodeoxynucleotide probe comprised of a modified RNA sequence attached to a DNA sequence attached to another modified RNA sequence. The modifications to the RNA sequences is to resist RNase-H—quite the opposite effect desired by Walder. Further, there is no suggestion or motivation provided by Inoue to modify its probe for use in antisense.

In the final rejection dated September 26, 2000, the Examiner essentially stated that the rejection in the previous Office Action was still applicable.

2. Claims 1-4, 12-14, 21-23, 31-33 and 41 are not obvious over the cited references.

A finding of obviousness under 35 U.S.C. §103 requires a determination of the scope and content of the prior art, the differences between the claimed invention and prior art, the level of ordinary skill in the art, and whether the differences are such that the claimed subject matter as a whole would have been obvious to one of ordinary skill in the art at the time the invention was made. *Graham v. Deere*, 383 U.S. 1 (1966). Where claimed subject matter has been rejected as obvious in view of a combination of prior art references, a proper analysis under §103 requires *inter alia*, consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success. *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991).

It has always been Appellants' view that the claimed invention was not obvious in view of the cited references, either singly or in combination. Although Walder et al. in the last paragraph does state that "it may be necessary to modify the oligonucleotide to decrease its rate of degradation and improve intracellular transport" and further states that the modified analogs need to retain normal hybridization properties and form substrates recognized and cleaved by RNase H, no teaching or direction is provided regarding how such analogs could be obtained. Specifically, there is no suggestion as to what modifications should be made and how modified analogs could retain retain normal hybridization properties and form substrates recognized and cleaved by RNase H. This is at best an "obvious to try"

situation. An 'obvious-to-try' situation exists when a general disclosure may pique the scientist's curiosity, such that further investigation might be done as a result of the disclosure, but the disclosure itself does not contain a sufficient teaching of how to obtain the desired result, or that the claimed result would be obtained if certain directions were pursued. *In re Eli Lilly & Co.*, 902 F.2d 943, 14 USPQ2d 1741 (Fed. Cir. 1990). It is well known that the "obvious to try" standard is clearly erroneous. *In re O'Farrell*, 7 USPQ2d 1673 (Fed. Cir. 1988). If anything, the disclosure of Walder et al. is further evidence of nonobviousness. Objective evidence such as commercial success, failure of others, long-felt need, and unexpected results must be considered before a conclusion on obviousness is reached. *Minnesota Mining and Manufacturing Co., v. Johnson & Johnson Orthopaedics Inc.*, 976 F.2d 1559, 24 USPQ2d 13221 (Fed. Cir. 1992).

The other two references, Ts'o et al.⁵ and Inoue et al. would not add much to the disclosure of Walder et al. Ts'o et al. primarily discloses compounds containing only methylphosphonate linkages. However, as stated above, compounds only containing methylphosphonate linkages are RNase H resistant. No direction is provided as to how many methylphosphonate linkages should actually be present. Other Miller et al. disclosures do not provide further guidance. For example, the Miller et al., 1985 references cited in the anticipation rejection discloses compounds containing all methylphosphonate linkages or 5'-phosphodiester linkage, followed by methylphosphonate linkages. Appellants note that other references authored by either of the inventors named in Ts'o et al., do not provide further guidance. Miller et al., 1980, J. Biol. Chem. 255:9659-9665 (**Tab 4**) and Miller et al., 1982, Biochemistry 21: 2507-2512 (**Tab 5**) disclose oligonucleotide analogs containing alternating

⁵ Referred to as Miller et al. in previous office actions

methylphosphonate/phosphodiester backbones. The Miller et al. references are silent regarding RNase sensitivity⁶.

Inoue et al. discloses an oligomer containing a 3-5 mer "joining with" 2'-O-methyl oligonucleotides at the 3' and 5' end. This oligomer was hybridized to ribooligonucleotide substrates. Inoue et al. disclosed that RNase H did not cleave 2'-O-Me-RNA/RNA hybrid, but did cleave the DNA/RNA hybrid. However, the disclosure of Inoue et al. was limited to 2'-O-methyloligonucleotides and could not possibly be extrapolated to other modifications.

3. Claims 8-9, 20, 27-28, 39 are not obvious over the cited references

Claims 8-9, which depend from claim 1 and claims 27-28 which depend from claim 21, recite that M are alkyl (claims 8 or 27) or methylphosphonate linkages (claims 9 and 28). Independent claims 20 and 39 also recite that M is a methylphosphonate moiety. None of these claims would be obvious for the reasons provided above.

4. The Cited References Do not disclose, much less suggest, many of the dependent claim limitations

The dependent claims are discussed below. The claims contain additional features neither disclosed nor suggested by the cited references. Therefore, the rejection of all of the following claims under 35 U.S.C. §103 are clearly improper and should be reversed.

⁶ It was disclosed in Furdon et al., 1989, Nucl. Acids Res. 17:9193-9204 (see abstract) that an oligonucleotide containing six methylphosphonate deoxynucleosides alternating with normal deoxynucleotides was RNase resistant.

Claims 5-7, 10-11, 15-19, 24-26, 29-30 and 34-38 are directed to specific embodiments. Specifically, claims 5-7 and claims 24-26 are directed to the bases, uracil, inosine, 2,6-diaminopurine; halogenated uracil or cytosine, substituted or unsubstituted 7-deazaguanine, 7-deazaadenine, 7-deazainosine, a methylated adenine, guanine, thymine or cytosine; claims 10-11 are directed to the following the internucleotide linkages, an alpha-phosphodiester 2' deoxynucleoside (claims 10 and 29), as well as an aminophosphonate, phosphotriester, phosphoramidate, carbamate or morpholino-substituted nucleotide (claims 11 and 30); claims 15-19 recite various "B" moieties, specifically, an intercalating agent, an isourea, a carbodiimide, an N-hydroxybenzotriazole (claims 15 and 24); a methylthiophosphonate (claims 16 and 25), a polypeptide or protein (claims 17 and 26) and a modified 2',3'-dideoxyribose nucleotide (claims 18 and 38) and claims 19 and 38 are directed to modified nucleotide where "y" is 2 or 3.

None of these specific embodiments are disclosed or suggested by the cited references. The Walder et al. reference merely discloses oligonucleotides and only suggests that it may be useful to make modifications; Ts'o et al. is only directed to methylphosphonates and Inoue et al. only teaches the use of 2'-O-methylribooligonucleotides at the 5' and 3' ends. There is absolutely no disclosure of modified bases recited in claims 5-7 and claims 24-26 in any of the cited references.

There is no disclosure of the internucleotide sequences, an alpha-phosphodiester 2' deoxynucleoside (claims 10 and 29), as well as an aminophosphonate, phosphotriester, phosphoramidate, carbamate or morpholino-substituted nucleotide (claim 11 and 30). Although Inoue et al. does disclose sequences disclosing 2'-O-methylribooligonucleotide at the 5' and 3' end, there is no other phosphotriester suggested. Furthermore, in order to put claim 11 in condition

for allowance or at least obviate some issues for appeal, Appellants have submitted a Second Amendment under 37 C.F.R. §1.116 in accordance with MPEP §1207 where it is proposed that claim 11 be amended to remove the recitation of "phosphotriesters". "Phosphotriester" is recited in new claim 53.

There is no disclosure or suggestion of the various "B" moieties recited in claims 15-18 and 34-37 in any of the cited references.

Finally, there is no suggestion of modified nucleotides in any of the cited references where there would be 2 or 3 "N" moieties at the 5' or 3' end.

Thus, the dependent claims would not be obvious over the cited references.

5. Claim 40 is not Obvious Over the Cited References

Claim 40 is directed to a method for identifying a nucleotide compound having a combination of nuclease resistance and ability to form an RNase H substrate when in complex with an RNA. Although Walder et al. does suggest that it would be desirable to modify a compound so that it has a decreased rate of degradation but retains RNase H sensitivity, there is no teaching regarding how such compounds could be modified. It would therefore follow that there is no suggestion or teaching of identifying such compounds. The other two cited references do not provide any further teachings. However, in order to put claim 40 in condition for allowance or at least obviate some issues for appeal, Appellants have submitted a Second Amendment under 37 C.F.R. §1.116 in accordance with MPEP §1207 where it is proposed that claim 40 be amended to recite that modified nucleotide compounds are prepared comprises at least one component selected from the group consisting of MN_3M , $B(N)_xM$ and $M(N)_xB$ wherein: N is a phosphodiester-linked modified or unmodified 2'-deoxynucleoside moiety; M is a moiety that confers endonuclease

resistance on said component and that contains at least one modified or unmodified nucleic acid base; B is a moiety that confers exonuclease resistance to the terminus to which it is attached; x is an integer of at least 2.

6. Claims 42-50 are not Obvious Over the Cited References

Claim 42 is directed to a compound containing at least two separate nuclease resistant components each consisting of two or more contiguous phosphodiester linked 2' deoxynucleosides, where one of the contiguous phosphodiester linked 2' deoxynucleosides is unmodified. Claims 43-49 depend from claim 42. Claim 50 recites that the two separate nuclease resistant sequences consist of 2 or 3 contiguous phosphodiester-linked 2'-deoxynucleosides.

None of the cited references disclose or suggest compounds containing at least **two** separate nuclease resistant components. The bulk of Walder et al. is directed to oligonucleotides. There is only a very general suggestion in Walder et al. regarding the utility of a compound having both nuclease resistance and RNase sensitivity. However, no further details are provided. Ts'o et al. only discloses methylphosphonate compounds. Inoue et al. discloses a compound containing 2'-O-methylribooligonucleotides at the 5' and 3' ends, not a compound containing **two** separate nuclease resistant components.

7. Claim 51 Is not Obvious over the cited References

Claim 51 is directed to a modified nucleotide compound and is actually a specific embodiment of the compound recited in claim 1. Specifically, in claim 51, "B" comprises a 2',3'-dideoxyribose. There is no disclosure or suggestion of such a

moiety in any of the cited references. Thus, claim 51 is not obvious over the cited references.

a. Claim 52 is not Obvious over the cited References

Claim 52 is directed to a modified nucleotide capable of forming RNase hybrids having at least one non-terminal moiety that confers nuclease resistance. As noted above, no direction is provided in Walder et al. regarding the location of a moiety conferring nuclease resistance; Ts'o et al. discloses only methylphosphonate compounds and Inoue et al. does not disclose any nuclease resistant moieties. Thus claim 52 is not obvious over the cited references.


IX. CONCLUSION

The present application contains an adequate written description of the claimed subject matter. Moreover, claims 1, 2, 4, 8, 12-14, 19 and 42-50 are not anticipated by Miller et al., 1985, Biochimie 67:769-776 and claims 1-4, 12-14 and 42-50 are not anticipated by Stein et al., 1988, Nucl. Acids Res. 16:3209-3221. Finally, claims 1-52 are not obvious over Walder et al., 1988, Proc. Natl. Acad. Sci. USA 85:5011-5015 in view of Ts'o et al., 1984, U.S. Pat. No. 4,469,863 and Inoue et al., 1987, Nucl. Acids Res. Symposium Series No. 18, pp. 221-224.

No extension or request or fee is believed due in connection with this filing, a Request for an extension of time (5 months) and authorization for the fee therefore having been filed therewith. In the event that any fee or fees are due, The Patent and Trademark Office is hereby authorized to charge the amount of any such fee(s) to deposit Account No. 05-1135, or to credit any overpayment thereto.

If a telephone conversation would further the prosecution of the present application, Applicants' undersigned attorney request that he be contacted at the number provided below.

Respectfully submitted,

A handwritten signature in cursive script that reads "Cheryl H. Agris".

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APPENDIX A-CLAIMS ON APPEAL

What Is Claimed Is:

1. A modified nucleotide compound which includes at least one component selected from the group consisting of MN_3M , $(N)_xM(N)_y$, $(N)_xM(N)_yM$, $B(N)_xM(N)_y$ and $(N)_xM(N)_yB$ wherein:
 - N is a phosphodiester-linked modified or unmodified 2'-deoxynucleoside moiety; provided that at least one N is a phosphodiester-linked unmodified 2' deoxynucleoside moiety;
 - M is a moiety that confers endonuclease resistance on said component and that contains at least one modified or unmodified nucleic acid base;
 - B is a moiety that confers exonuclease resistance to the terminus to which it is attached;
 - x is an integer of at least 2; and
 - y is an integer.
2. The modified nucleotide compound of claim 1 wherein M and B are the same moiety.
3. The modified nucleotide compound of claim 1 which, when in complex with a complementary RNA, confers RNase H sensitivity to the RNA.
4. The modified nucleotide compound of claim 1 wherein N contains at least one adenine, guanine, thymine or cytosine moiety.

5. The modified nucleotide compound of claim 1 wherein N contains at least one uracil, inosine or 2, 6-diaminopurine moiety.
6. The modified nucleotide compound of claim 1 wherein N contains at least one 5-halogenated uracil or cytosine or a substituted or unsubstituted 7-deazaguanine, 7-deazaadenine or 7-deazainosine moiety.
7. The modified nucleotide compound of claim 1 wherein N contains at least one methylated adenine, guanine, thymine or cytosine moiety.
8. The modified nucleotide compound of claim 1 wherein M is a $C_1 - C_4$ alkylphosphonate deoxynucleotide.
9. The modified nucleotide compound of claim 8 wherein M is a methylphosphonate deoxynucleotide.
10. The modified nucleotide compound of claim 1 wherein M is an alphaphosphodiester 2'deoxy nucleoside.
11. The modified nucleotide compound of claim 1 wherein M is selected from the group consisting of an aminophosphonate, phosphotriester, phosphoramidate, carbamate or morpholino-substituted nucleotide.
12. The modified nucleotide compound of claim 1 wherein B is directly or indirectly attached to the deoxyribose moiety of at least one of the 3'-and 5'-terminal nucleotides.

13. The modified nucleotide compound of claim 12 wherein B is directly or indirectly attached to a hydroxyl group of the deoxyribose of at least one of the 3'-and 5'- terminal nucleotides.
14. The modified nucleotide compound of claim 12 wherein B is directly or indirectly attached to a phosphate moiety attached to the deoxyribose moiety of at least one of the 3'- and 5'- terminal nucleotides.
15. The modified nucleotide compound of claims 13 or 14 wherein B is selected from the group consisting of an intercalating agent, an isourea a carbodiimide and an N-hydroxybenzotriazole.
16. The modified nucleotide compound of claim 13 wherein B is a methylthiophosphonate.
17. The modified nucleotide compound of claims 13 or 14 wherein B is a polypeptide or protein.
18. A modified nucleotide compound of claim 1 which includes at least one sequence of the formula $(N)_y M(N)_x B$ wherein B is a modified or unmodified 2', 3'-dideoxyribose nucleotide.
19. The modified nucleotide compound of claim 1 wherein y is an integer selected from the group consisting of 2 or 3.

20. A modified nucleotide compound which contains at least one sequence having the formula MN_3M wherein N is a phosphodiester-linked unmodified 2'-deoxynucleoside moiety containing at least one guanine, adenine, cytosine or thymine moiety and M is a methylphosphonate-containing deoxynucleotide.

21. A method of inhibiting the function of an RNA, which comprises: contacting said RNA, under conditions permissive of hybridization, with a modified nucleotide compound which includes at least one complimentary component selected from the group consisting of MN_3M , $(N)_xM(N)_y$, $(N)_xM(N)_yM$, $B(N)_xM(N)_y$ and $(N)_xM(N)_yB$ wherein:

N is a phosphodiester-linked modified or unmodified 2'-deoxynucleoside moiety;

M is a moiety whose presence confers endonuclease resistance on said component and that contains at least one modified or unmodified nucleic acid base;

B is a moiety whose presence confers exonuclease resistance to the terminus to which it is attached; and

x is an integer of at least 2.

22. The method of claim 21 wherein the RNA is contacted with a compound wherein M and B are the same moiety.

23. The method of claim 21 wherein the RNA is contacted with a compound wherein N contains at least one adenine, guanine, thymine or cytosine moiety.

24. The method of claim 21 wherein the RNA is contacted with a compound wherein N contains at least one uracil, inosine or 2, 6-diaminopurine moiety.
25. The method of claim 21 wherein the RNA is contacted with a compound wherein N contains at least one 5-halogenated uracil or cytosine or a substituted or unsubstituted 7-deazaguanine, 7-deazaadenine or 7-deazainosine moiety.
26. The method of claim 21 wherein the RNA is contacted with a compound wherein N contains at least one methylated adenine, guanine, thymine or cytosine moiety.
27. The method of claim 21 wherein the RNA is contacted with a compound wherein M is a C₁-C₄ alkylphosphonate.
28. The method of claim 27 wherein the RNA is contacted with a compound wherein M is a methylphosphonate.
29. The method of claim 21 wherein the RNA is contacted with a compound wherein M is an alpha-phosphodiester 2'deoynucleoside.
30. The method of claim 21 wherein the RNA is contacted with a compound wherein M is selected from the group consisting of an aminophosphonate, phosphotriester, phosphoramidate, carbamate or morpholino-substituted nucleotide.

31. The method of claim 21 wherein the RNA is contacted with a compound wherein B is directly or indirectly attached to the deoxyribose moiety of at least one of the 3'-and 5'- terminal nucleotides.
32. The method of claim 31 wherein the RNA is contacted with a compound wherein B is directly or indirectly attached to a hydroxyl group of the deoxyribose of at least one of the 3'- and 5' terminal nucleotides.
33. The method of claim 31 wherein the RNA is contacted with a compound wherein B is directly or indirectly attached to a phosphate group attached to the deoxyribose moiety of at least one of the 3'-and 5'- terminal nucleotides.
34. The method of claims 32 or 33 wherein the RNA is contacted with a compound wherein B is selected from the group consisting of an intercalating agent, an isourea, a carbodiimide and an N-hydroxybenzotriazole.
35. The method of claim 32 wherein the RNA is contacted with a compound wherein B is a methylthiophosphonate.
36. The method of claims 32 or 33 wherein the RNA is contacted with a compound wherein B is a polypeptide or protein.
37. The method of claim 21 wherein the RNA is contacted with a compound which includes at least one sequence of the formula $(N)_y M(N)_x B$ wherein B is modified or unmodified 2', 3'-dideoxyribose nucleotide.

38. The method of claim 21 wherein the RNA is contacted with a compound wherein x is selected from the group consisting of 2 or 3.

39. The method of claim 21 wherein the RNA is contacted with a modified nucleotide compound which includes at least one sequence having the formula MN_3M wherein N is a phosphoester-linked unmodified 2'-deoxynucleoside moiety containing at least one guanine, adenine, cytosine or thymine moiety and M is a methylphosphonate-containing deoxynucleoside.

40. A method of identifying a nucleotide compound having a combination of nuclease resistance and the ability to form an RNase H substrate when in complex with an RNA, which method comprises:

- (i) preparing modified nucleotide compounds;
- (ii) selecting by exo-and endonuclease digestion those modified nucleotide compounds of (i) which are nuclease-resistant as shown by being capable of forming and electrophoretically migrating as a duplex with a complementary nucleotide compound; and
- (iii) selecting by RNase H digestion those of the nuclease-resistance nucleotide compounds of (ii) which act as substrates for RNase H when hybridized with a complementary RNA.

41. A method of treating a human or animal so as to inhibit the function of a target RNA therein which method comprises administering a therapeutically effective amount of a modified nucleotide compound so as to inhibit the function of the target RNA, which modified nucleotide compound includes at least one component selected from the group consisting of MN_3M , $(N)_xM(N)_y$, $(N)_xM(N)_yM$, $B(N)_xM(N)_y$ and $(N)_xM(N)_yB$; wherein N is a phosphodiester-linked modified or

unmodified 2'-deoxynucleoside moiety, M is a moiety that confers endonuclease resistance on said component and that contains at least one modified or unmodified nucleic acid base, B is a moiety that confers exonuclease resistance to the terminus to which it is attached, x is an integer of at least 2, and y is an integer.

42. A compound containing at least 2 separate nuclease resistant components each consisting of 2 or more contiguous phosphodiester-linked 2' deoxynucleosides; wherein at least one of said contiguous phosphodiester-linked 2' deoxynucleosides is unmodified.

43. The compound of claim 42 which is capable of specifically binding with a nucleic acid sequence of interest to inhibit the function thereof.

44. The compound of claim 42 which, when complexed with a complementary RNA, confers RNase H sensitivity upon the RNA.

45. The compound of claim 42 which comprises an oligonucleotide or polynucleotide.

46. The compound of claim 45 wherein the oligonucleotide or polynucleotide is modified.

47. The compound of claim 46 wherein the modified oligonucleotide or polynucleotide consists of at least one moiety which confers endonuclease resistance and at least one moiety which confers exonuclease resistance.

48. The compound of claim 47 wherein the endonuclease-resistance conferring moiety also confers exonuclease resistance to the modified nucleotide component.

49. The compound of claim 47 wherein the portion of the compound that can function as an RNase H substrate is located between the moiety conferring exonuclease resistance and the moiety conferring endonuclease resistance.

50. A compound containing at least two separate nuclease resistant sequences which consists of 2 or 3 contiguous phosphodiester-linked 2'-deoxynucleosides; wherein at least one of said contiguous phosphodiester-linked 2' deoxynucleosides is unmodified.

51. A modified nucleotide compound which comprises at least one component selected from the group consisting of MN_3M , $(N)_xM(N)_y$, $(N)_xM(N)_yM$, $B(N)_xM(N)_y$ and $(N)_xM(N)_yB$ wherein:

N comprises a phosphodiester-linked modified 2' deoxynucleoside moiety;

M is a moiety that confers endonuclease resistance on said component and which contains at least one nucleic acid base with a 3' methoxylphosphonate;

B is a moiety that confers exonuclease resistance to the terminus to which it is attached and comprises a 2', 3'-dideoxyribose nucleotide;

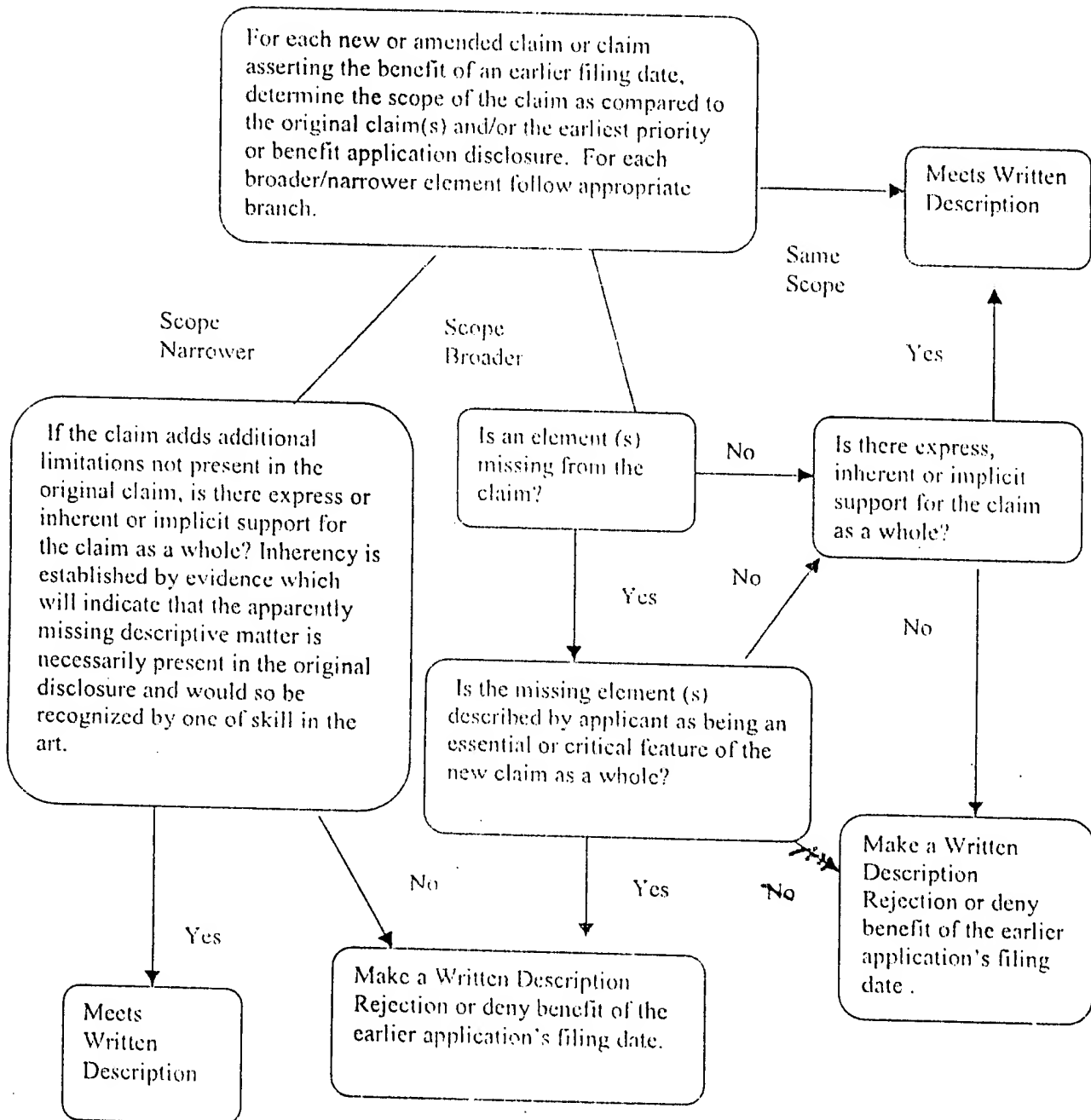
x is an integer of about 2; and

y is an integer.

52. A modified nucleotide compound capable of forming RNase H sensitive hybrids and having improved nuclease resistance comprising at least one non-terminal moiety that confers nuclease resistance on said compound and contains at least one modified or unmodified nucleic acid base and at least one non-terminal phosphodiester-linked unmodified 2' deoxynucleoside moiety.

Written Description Amended
or New Claims, or Claims Asserting
the Benefit of an Earlier Filing Date

Decision Tree



Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides

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ABSTRACT

We have studied the translation of rabbit globin mRNA in cell free systems (reticulocyte lysate and wheat germ extract) and in microinjected *Xenopus* oocytes in the presence of anti-sense oligodeoxynucleotides. Results obtained with the unmodified all-oxygen compounds were compared with those obtained when phosphorothioate or α -DNA was used. In the wheat germ system a 17-mer sequence targeted to the coding region of β -globin mRNA was specifically inhibitory when either the unmodified phosphodiester oligonucleotide or its phosphorothioate analogue were used. In contrast no effect was observed with the α -oligomer. These results were ascribed to the fact that phosphorothioate oligomers elicit an RNase-H activity comparable to the all-oxygen congeners, while α -DNA/mRNA hybrids were a poor substrate. Microinjected *Xenopus* oocytes followed a similar pattern. The phosphorothioate oligomer was more efficient to prevent translation than the unmodified 17-mer. Inhibition of β -globin synthesis was observed in the nanomolar concentration range. This result can be ascribed to the nuclease resistance of phosphorothioates as compared to natural phosphodiester linkages. α -oligomers were devoid of any inhibitory effect up to 30 μ M. Phosphorothioate oligodeoxyribonucleotides were shown to be non-specific inhibitors of protein translation, at concentrations in the micromolar range, in both cell-free systems and oocytes. Non-specific inhibition of translation was dependent on the length of the phosphorothioate oligomer. These non-specific effects were not observed with the unmodified or the α -oligonucleotides.

INTRODUCTION

The use of antisense oligodeoxynucleotides as specific inhibitors of gene expression has undergone a rapid expansion over the past several years (1,2). Central to this approach is the presumption that messenger RNAs bound as RNA-DNA duplexes either cannot be translated by ribosomes (3-5) or are destroyed by RNase-H (6-10). In order to be effective *in vivo*, synthetic oligonucleotides must share several properties. Among these are :1) chemical stability, 2) water solubility, 3) high thermodynamic stability of the RNA-DNA duplex and 4) nuclease resistance. Unmodified phosphodiester (PO) DNA meets these requirements except for the last one. Thus, a series of modified derivatives have recently been synthesized (11-14). Substitution of sulfur for one of the phosphodiester oxygen atoms yields a molecular species that meets all four criteria (12). These phosphorothioate (PS) oligodeoxynucleotides have indeed recently been shown to inhibit the cytopathic effect of HIV-1 (15; Matsukura et al., unpublished results). In experiments in chronically infected H9 cells, the expression of p24 gag protein was shown to fall >90% in the presence of a 10 μ M concentration of a

phosphorothioate sequence complementary to the 5' region of the *rev* (formerly *art/hrs*) gene. The normal congener was ineffective as was a methylphosphonate construct. Marcus-Sekura et al. (16) showed that an anti-sense phosphorothioate was an effective inhibitor of chloramphenicol acetyl transferase activity in the standard CAT assay. In a series of experiments conducted in HL60 cells, an anti-sense *c-myc* phosphorothioate oligomer was unable to consistently inhibit cellular proliferation unless supplied in liposomes, whereas in multiple experiments, the normal oligomer inhibited levels of myc protein by >50% at 12 h (17).

Another class of modified oligonucleotide meets the criterion of nuclease resistance. In these compounds the natural β -configuration of the nucleoside is transposed into its α -analogue (13, 14, 18-26). However these α -oligonucleotides were reported to be poor inhibitors of VSV mRNA translation in rabbit reticulocyte lysate (27).

Cell-free systems (28-34) and micro-injected *Xenopus* oocytes (8,35-37) are effective means for evaluating the ability of modified oligonucleotides to act as antisense inhibitors. We present here a comparative study of both unmodified and nuclease resistant oligonucleotides tested for their ability to promote selective arrest of rabbit globin mRNA translation.

MATERIALS AND METHODS

Oligodeoxynucleotides

Phosphodiester oligodeoxynucleotides were synthesized either on a Pharmacia or on an Applied Biosystems Model 380B Synthesizer, and were purified via high-pressure liquid chromatography (Waters) on a PRP-1 column. Phosphorothioates were synthesized and purified via a modification (12) of the procedure of Stec et al. (38). Alpha-oligodeoxynucleotides were synthesized on a Pharmacia automatic synthesizer and purified as previously described (24). The oligomer length homogeneity was periodically evaluated by running samples on 15% polyacrylamide/6M urea gels. After electrophoresis, bands were either stained with ethidium bromide and viewed by UV-light or revealed by autoradiography in the case of ^{32}P -labelled oligonucleotides. All preparations yielded a single species in each lane loaded.

Cell-free translation systems

Wheat germ extract was purchased either from New England Nuclear or from Genofit (Geneva). Oligomer was added to a translation mixture containing ^{35}S -methionine. Unless otherwise stated experiments in wheat germ extracts were performed under the following conditions: 0.05 μg of rabbit globin mRNA was mixed with the oligonucleotide and added to 30 μl of the translation mixture. The final concentration of total mRNA was 9.3nM (i.e. 3.9nM in β -globin). The reaction was run at 25°C during 30 min. Reactions were generally carried out without premixing RNA with the oligomer. We showed that premixing did not alter the results.

Rabbit reticulocyte lysate was purchased from New England Nuclear. The oligomer, at

the appropriate concentration, was added to the translation mixture (25 μ l) containing 0.1 μ g globin mRNA and 35 S-methionine. The samples were then incubated for 90 min at 37°C.

An aliquot of the reaction mixtures was then analyzed either on a 15% polyacrylamide-SDS gel with a 5% stack or on a 12% polyacrylamide gel containing 8 mM Triton X100 and 6M urea. The gels were then fixed in a 40% methanol/7% acetic acid solution for about 1h, soaked in a solution of sodium salicylate ("Fluoro-Hance", Research Products International Corp.) for 30 min, and dried under vacuum prior to autoradiography.

Translation in *Xenopus* oocytes

Stage 6 oocytes (selected via stereotactic microscopy) were obtained from the Laboratoire de Physiologie de la Reproduction (Paris VI University). Specimens were maintained in modified Barth's saline solution (39). 80 nl of a 1/1 (v/v) mixture of globin mRNA (50 μ g/ml) and oligomer, dissolved in sterile distilled water, were injected in *Xenopus* oocytes; 4 to 5 hours after injection, oocytes were incubated in the presence of 35 S-methionine for about 15 hours. The samples were then homogenized in 20 μ l (per oocyte) of 20 mM Tris, pH 7.6, 0.1 M NaCl, 1% Triton X100 and 1 mM PMSF (40). Proteins were then analysed by SDS-PAGE electrophoresis on a 13.5% acrylamide gel. Assuming a free diffusion compartment of 0.5 μ l inside the oocyte, the final intracellular concentration of β -globin mRNA was about 16 nM.

Hybridization studies

1 μ g of rabbit globin mRNA was bound to a nitrocellulose filter by heating at 80°C during 2 hours. The filter was incubated in a mixture containing about 10^7 cpm of 32 P-labelled oligomer and 2 ml of 6xSSC/10xDenhardt's solution (1xSSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2; 10xDenhardt's is 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinyl-pyrrolidone). The filters were then placed in a thermostated holder, and were eluted with 6xSSC as the temperature was increased at a rate of 1.2°C/min. Thermal elution profiles were constructed, and the T_c determined to be that temperature at which 50% of the total counts had been eluted (41).

Oligodeoxynucleotide-promoted cleavage of globin mRNA by RNase-H

RNase-H from *E. coli* was obtained from Genofit. Kinetic experiments were run at 37°C in a 20 mM Tris-HCl buffer, pH 7.5 containing 100 mM KCl, 10 mM MgCl₂ and 0.1 mM dithiothreitol. Incubations of 0.3 μ g of rabbit globin mRNA and 30pmoles of oligonucleotide were performed in a total volume of 30 μ l, in the presence of 2.5 units of enzyme. At the appropriate times aliquots of 5 μ l were spotted on a nylon membrane. UV-irradiated membranes were probed with 32 P 5' end-labelled 17PO- β (Figure 1) and autoradiographed.

RESULTS

Sequences studied

The sequences of the oligonucleotides used throughout this study and of the complementary regions on rabbit α - and β -globin mRNAs are given in figure 1. We targeted

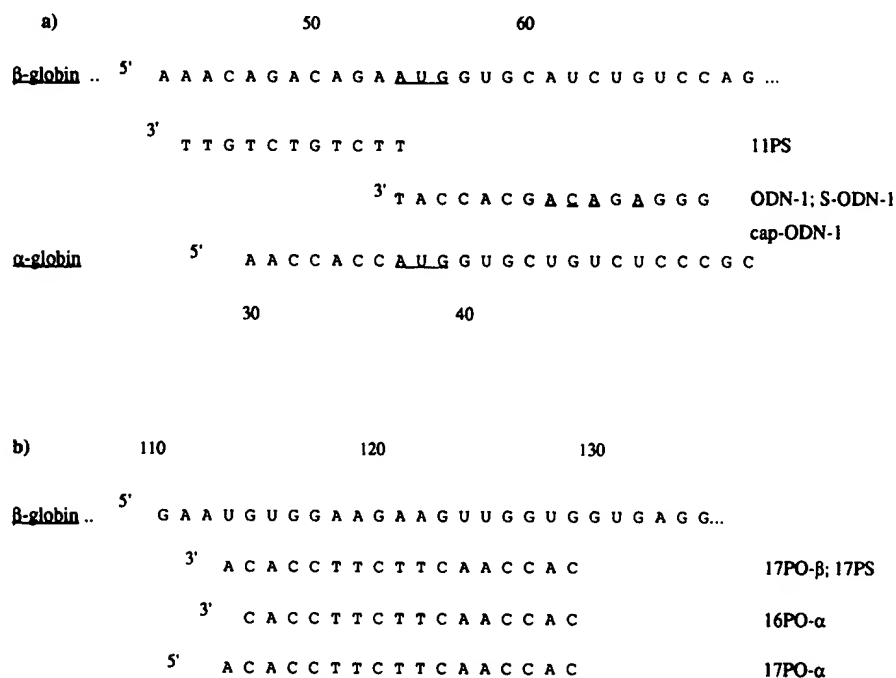


Figure 1 : Nucleotide sequences of oligodeoxynucleotides complementary to a) AUG region of rabbit β-globin and α-globin (51) and b) coding sequence of rabbit β-globin mRNA. The numbering above the RNA sequences refer to the transcription start; the translation initiation codons are underlined. The abbreviations of the antisense oligonucleotides are indicated on the right of the sequences (for details see "Results; Sequences studied"). 11-mers, 17-mers and the 16-mer were complementary to the β-globin mRNA and ODN-1 to the α-globin mRNAs. The latter oligonucleotide can pair with the β-message giving 4 mismatches (underlined letters in the ODN-1 sequence).

two regions of the β-globin mRNA, namely nucleotides 44-54 and 113-129 that were already selected in a previous study (8). The 44-54 sequence is located immediately upstream of the start codon, while the 113-129 sequence is within the coding region of the message. An unmodified phosphodiester (PO) oligonucleotide (17PO-β) and phosphorothioate (PS) analogues (11PS; 17PS), complementary to these two regions, were synthesized. Two α-oligodeoxynucleotides complementary to the coding region were also used. The first one, 16PO-α, was designed to be in an antiparallel orientation with respect to the target sequence. A second one, 17PO-α was synthesized to bind its target in a parallel orientation.

In addition we constructed several oligomers complementary to the α-globin mRNA sequence. ODN-1 is a 15-mer sequence complementary to the α-globin initiation codon and downstream region (Figure 1). This sequence also complements a similar region of the β-globin message with the exception of a 4-base mismatch, assuming the formation of two G-U

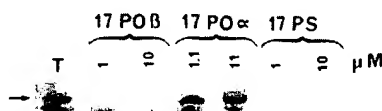


Figure 2 : Effect of various 17-mers on in vitro synthesis of rabbit β -globin. Autoradiograph of a 12% Triton-acetic acid-urea polyacrylamide gel of proteins synthesized in wheat germ extracts as indicated in Materials and Methods in the absence (T) or in the presence of 17PO- β , 17PO- α or 17PS at the indicated concentration. The upper band (arrow) corresponds to β -globin and the lower one to α -globin.

pairs. ODN-1[Sen] is the sense construct. ODN-1 and ODN-1[Sen] were also synthesized in all-phosphorothioate forms (S-ODN-1; S-ODN-1[Sen]). In addition, they were also constructed with two phosphorothioates at both the 3' and 5' ends, and are referred to as cap-ODN-1 and cap-ODN-1[Sen], respectively.

A series of random sequences were also made. ODN-2 (5'-dACTCC-3') is a 5-mer, and ODN-3 (5'-dCCAAACCATG-3') a 10-mer. ODN-2 and -3 were synthesized as phosphorothioate derivatives only. A random 16-mer, termed ODN-4, with base composition equivalent to ODN-1 (5'-dACGCGAGGACCATAGT-3') contained 5 contiguous phosphorothioates at the 5' and 3' ends, separated by 5 phosphodiester linkages. We also synthesized the same 16-mer (5'-dACGCGAGGACCATAGT-3') containing alternating PS and PO linkages (ODN-5). The absence of perfect complementarity between these oligomers and rabbit globin mRNAs was checked by a computer search (CITI 2).

Table 1

Oligos	Tc	WGE	Xenopus oocytes		
			oligo/RNA	Coinjection	RNA/oligo
17PO- β	60	50	>>	30	650
17PO- α	50	>>		>>	
17PS	39	50	30	3	7
11PS	28	3000		8000	

Table 1 : Stability of oligonucleotide/RNA hybrids and inhibition of translation by antisense oligonucleotides. Temperature of half-dissociation (Tc) of filter-bound complexes, determined as described in Material and Methods are given in °C. Concentrations (indicated in nM), leading to a 50% decrease of translation either in wheat germ extracts (WGE) or in oocytes following co-injection or delayed injections (RNA/oligo means RNA first; oligo/RNA means oligo first; see text) were determined from curves shown on figures 3 and 6. The symbol >> indicates that 50% inhibition was not reached at the highest concentration tested.

Effects of oligonucleotides on translation in cell-free media

Translation in wheat germ extract : Translation of rabbit globin mRNA in cell-free systems gives rise to two bands corresponding to the α - and β -chains which can be separated on Triton-urea-acetic acid polyacrylamide gels. In a first set of experiments we compared the effects on globin synthesis of three 17-mers, 17PO- β , 17PO- α and 17PS, targeted to the coding region of β -globin mRNA (Figure 1). The results shown in figure 2 indicate that 17PO- β is a specific anti-sense inhibitor in a wheat germ system: a fifty per cent decrease was observed at 50 nM (Table 1) and a total inhibition of β -globin synthesis was attained at 1 μ M, whereas α -globin synthesis was not affected, in good agreement with a previous report (8). In contrast even at 11 μ M no effect was observed in the presence of 17PO- α . At low concentration (below ca. 1 μ M) a specific decrease of β -globin synthesis resulted from addition of 17PS to the translation mixture, 50% inhibition being observed at 50 nM as in the case of 17PO- β (Table 1). However, by 1-2 μ M, the synthesis of α -globin was also decreasing and at 10 μ M 17PS both α - and β -globin mRNA synthesis were 100% inhibited (Figure 2). As this oligonucleotide is not complementary to any region of α -globin mRNA (no match above than 70% homology) this should be ascribed to a non-specific effect on translation. Thus, in a defined concentration range (<500nM), the anti-sense 17PS inhibits the synthesis only of its directed target, i.e., β -globin; and the sense analogue, in that same concentration range, has little if any effect on the synthesis of either α - or β -globin (data not shown).

A similar conclusion regarding antisense specificity can be drawn from experiments with 11PS, a phosphorothioate oligomer complementary to the region immediately upstream of the AUG codon of β -globin (Figure 1), homologous to an unmodified 11-mer (11PO) that we used in a previous study (8). Specific inhibition of β -globin synthesis (at low concentration) and non-specific inhibition of α -globin mRNA translation (at high concentration) were observed but 11PS was a less efficient inhibitor than 11PO. It should also be noted that higher concentrations of 11PS (>5 μ M) than those of 17PS were required to observe non-specific effects (Figure 3). This can be related to a length effect (see below).

We also tested ODN-1, a 15-mer complementary to the α -message (Figure 1), in the wheat germ system; 100% inhibition of translation was achieved at 1 μ M (Figure 4) but no inhibition was observed with the sense congener (data not shown). On the other hand, both sense and anti-sense S-ODN-1 constructs were potent inhibitors of translation at 5 μ M, with control levels being reached at ca. 500 nM (Figure 4). The region from 500 nM to 5 μ M was not examined in greater detail in this study. Translation of Brome Mosaic Virus mRNA was also inhibited, in the wheat germ system, in the presence of S-ODN-1[Sen], to which it has no sequence homology above 70% (Figure 4).

We evaluated the dependence of non-specific translation inhibition on phosphorothioate oligomer length. The random 5-mer ODN-2 was not inhibitory to globin mRNA translation up to 25 μ M and only partially inhibitory at higher concentrations (Figure 4). If the random

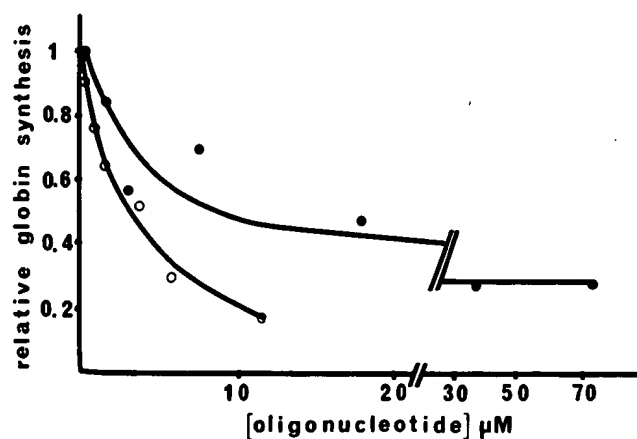


Figure 3 : Effect of 11PS on rabbit β -globin synthesis. Globin mRNA was either translated in wheat germ extracts (O) or in micro-injected *Xenopus* oocytes (●). ^{35}S -labelled proteins were analyzed by gel electrophoresis (see Materials and Methods). β -globin synthesis was determined from densito-meter tracings of autoradiographs, relatively to the synthesis observed in the absence of added oligodeoxynucleotide.

10-mer, (ODN-3), was used in the translation assay, control levels of protein synthesis were not achieved at concentrations higher than $3 \mu\text{M}$. ODN-3 has no complementary sequence matching better than 70%, neither in α - nor in β -globin mRNA. In the case of ODN-1 several sequences were found that could form four base pairs. Such hybrids are not expected to be stable under our conditions. A 28-mer, S-dC₂₈, was also examined in the wheat germ system. This oligomer is capable of inhibiting the cytopathic effect of the HIV virus in newly infected H9 cells, at concentrations in the low micromolar range (15). S-dC₂₈ was the most potent inhibitor tested in this system: translation of globin mRNA was entirely inhibited above 500 nM.

As the non-specific inhibitory properties of phosphorothioate oligodeoxynucleotides appeared to be highly length dependent, we wanted to determine if a molecule containing blocks of contiguous phosphorothioates (where each block was itself too short to be an inhibitor) could act in summation to produce an effective translation inhibitor. Because we knew that a 5-mer phosphorothioate had a limited inhibitory effect on translation, we synthesized a random 16-mer ODN-4, with base composition essentially equivalent to ODN-1, containing 5 contiguous phosphorothioates at the 5' and 3' ends separated by 5 phosphodiester linkages. When tested in the wheat germ system, 100% inhibition of translation was seen at concentrations higher than $6.25 \mu\text{M}$ (Figure 4). Thus, this molecule does not behave as if it were two separated 5-mers, but rather as if it were a 10-mer (compare to ODN-3).

The non-specific inhibition of translation is not related only to the length of the

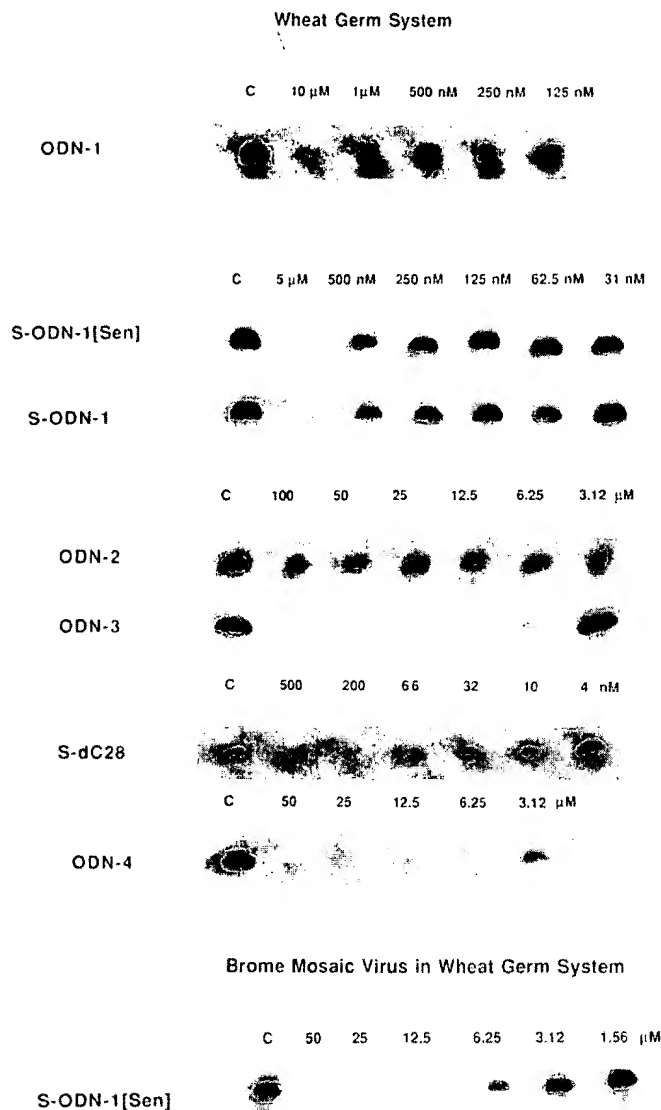


Figure 4 : Translation of rabbit globin mRNA and Brome Mosaic Virus mRNA in the wheat germ system in the presence of various oligomers: ODN-1 is a 15-mer complementary to α -globin mRNA; S-ODN-1 is the phosphorothioate analogue; ODN-1[Sen] and S-ODN-1[Sen] are the sens oligomers; cap-ODN-1 contains two phosphorothioate linkages at the 5'- and at the 3'-ends; ODN-2 and ODN-3 are random phosphorothioate 5-mer and 10-mer, respectively; ODN-4 is a random 15-mer composed of two blocks of 5 phosphorothioates separated by 5 phosphodiester (for more details see "Results; Sequences studied"). C=control (no added oligomer). Numerals above each lane are the concentration of added oligomer (in μ M or in nM). Incubation time was 120 min. at 22°C.

oligomer: cap-ODN-1 (antisense) also inhibited protein translation (100% below 3 μM) while the sense congener exhibited dose dependent inhibition above 3 μM (data not shown). This non-specific inhibition was unexpected as the molecule contained only two phosphorothioates at each end. In contrast, a concentration of 100 μM ODN-5, a random 16-mer with essentially the same base composition as ODN-1 and containing eight alternating phosphorothioates, was required for 100% inhibition of translation (data not shown).

Translation in rabbit reticulocyte lysate : When ODN-1 was used in the reticulocyte lysate system, concentration dependent inhibition of globin synthesis was observed, with 100% inhibition (α - plus β -) seen at 100 μM , while no inhibition was seen for the sense analog (5'-dATGGTGCTGTCTCCC-3') up to 100 μM (Figure 5). This is approximately a 100-fold decrease in sensitivity as compared to the wheat germ extract, and may reflect low levels of RNase-H activity present in reticulocyte lysate (7). However, when phosphorothioates of identical sequence were used under similar reaction conditions, both the sense and the antisense 15-mers were 100% inhibitory (α - plus β -globin) above 6.25 μM (Figure 5). Note that the sense construct appears to be even more inhibitory than the anti-sense species below 6.25 μM . This observation precludes the existence, under these reaction conditions, of even a narrow concentration range, or "window", of anti-sense specificity in the rabbit reticulocyte system.

The effect of S-oligomer length on inhibition of translation was also evaluated in the reticulocyte lysate system. ODN-2, a random 5-mer, was not inhibitory at any concentration tested (up to 100 μM), while ODN-3, the random 10-mer, was inhibitory (α - plus β -globin) at concentrations above 25 μM . S-dC₂₈ and its phosphodiester congener O-dC₂₈ were tested. They both bind to the reverse transcriptase of HIV-1 (50) but O-dC₂₈ has a lower affinity than S-dC₂₈. In the reticulocyte lysate system, O-dC₂₈ did not inhibit protein translation at 100 μM , while S-dC₂₈ was virtually completely inhibitory at concentrations higher than 3 μM (Figure 5).

Variants of ODN-1, which contains two phosphorothioates only at the 3' and 5' end, were also examined in the reticulocyte lysate system. For the antisense construct (cap-ODN-1), a dose dependent inhibition of protein translation was observed, with 100% inhibition (α - plus β -globin) seen at 100 μM (Figure 5). Control levels of translation were achieved below 25 μM . In experiments with the sense construct (cap-ODN-1{Sen}), no inhibition was seen until a concentration of 100 μM was obtained. Thus, in this system, there appears to be a window of antisense specificity in the 25-100 μM concentration range.

Xenopus oocytes :

Following micro-injection, β -globin mRNA is efficiently translated in *Xenopus* oocytes (the synthesis of the α -polypeptide requires the presence of hemin). Figure 6 displays the results of the gel electrophoresis of oocyte proteins after co-injection of rabbit globin mRNA with two different concentrations of both normal (17PO- β), α (17PO- α) and phosphorothioate (17PS) oligomers. Results similar to those in the wheat germ system were obtained. 17PO- α

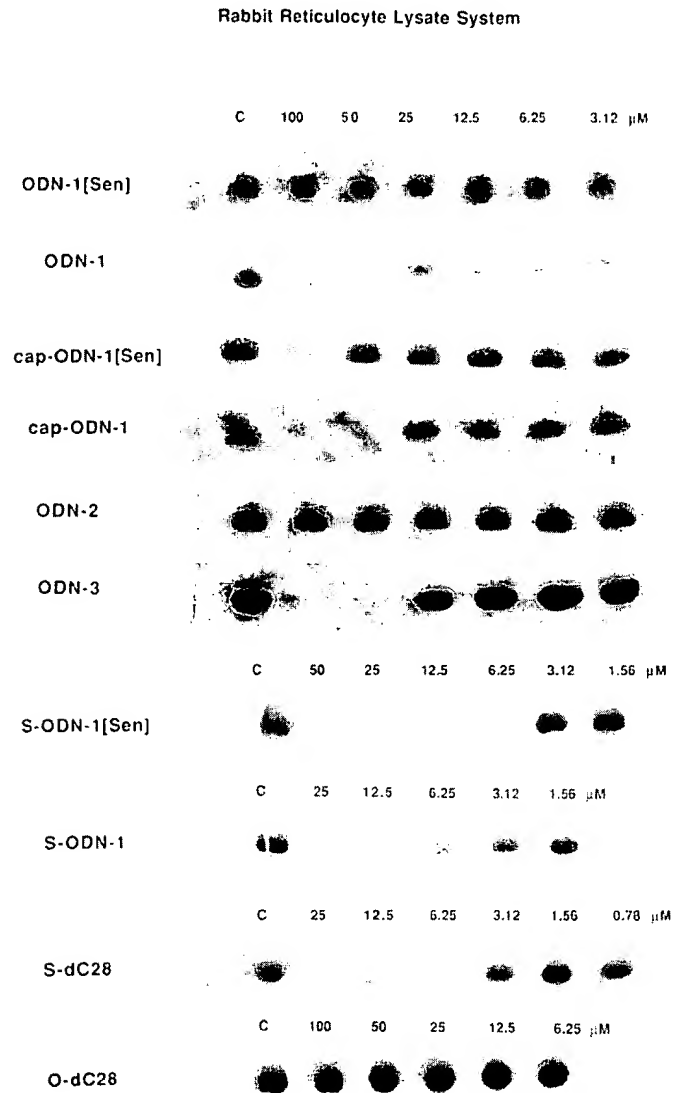


Figure 5 : Translation of rabbit globin mRNA in the reticulocyte lysate system in the presence of various oligomers (see legend of figure 4). C=control (no added oligomer). Numerals above each lane are the concentration of added oligomer (in μ M or in nM). Incubation time was 90 min. at 37°C.

did not inhibit β -globin synthesis at either concentration (3.2 μ M or 16 μ M, lanes 3 and 6). Both 17PO- β and 17PS were partially inhibitory at low concentration (lanes 7 and 8). At higher concentration (16 μ M) 17PO- β selectively inhibited the production of β -globin. In

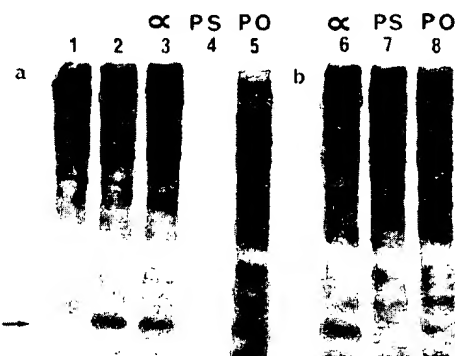


Figure 6 : Effect of various 17-mers on the synthesis of rabbit β -globin in micro-injected *Xenopus* oocytes. Autoradiograph of a 12.5% polyacrylamide-SDS gel of proteins synthesized in oocytes injected with rabbit globin mRNA in the absence (lane 2) or in the presence of 17PO- α (lanes 3 and 6), 17PS (lanes 4 and 7), or 17PO- β (lanes 5 and 8), at a concentration of 16 μ M (panel a lanes 2-5) or 3.2 μ M (panel b, lanes 6-8). Lane 1 corresponds to non injected oocytes. The arrow indicates the position of β -globin.

contrast, global protein synthesis was completely prevented by 17PS at this latter concentration (lane 4). This result was reminiscent to the non-specific effect induced by this oligomer in the wheat germ extract.

When the oligomers were co-injected with the message, the ability of 17PS to specifically inhibit β -globin production was greater than that of its oxygen analogue at similar concentrations (Figure 7 and Table 1). This may be due in part to the decreased sensitivity of this compound to nucleases (12). The effect of nuclease resistance on translation inhibition was better seen when a delay was introduced between injections of the oligomer and of the globin mRNA. Specific inhibition of β -globin synthesis still occurred when 17PS was injected 6 hours prior to the message although it was less efficient than upon co-injection. The concentrations leading to 50% reduction were 30 and 3 nM respectively (Figure 7b and Table 1). In contrast no effect was detected when 17PO- β was injected 6 hours prior to mRNAs even when the oligomer concentration was as high as 3 μ M (Figure 7a).

In order to test a more physiological situation in which the mRNA was already engaged in translation (as is the case for endogenous RNAs from the oocyte), 17-mers were injected 6 hours after globin mRNA. Previous experiments have shown that within 6 hours after microinjection, globin mRNA is recruited into polysomes and efficiently translated. Specific inhibition of β -globin synthesis was observed in the nanomolar range with 17PS whereas 100 fold higher concentrations of 17PO- β were required for half-inhibition (Figure 7 and Table 1).

In fair agreement with what was observed in the wheat germ extract, 11PS was much less inhibitory (3 orders of magnitude) in oocytes compared to 17PS. Concentrations in the μ M

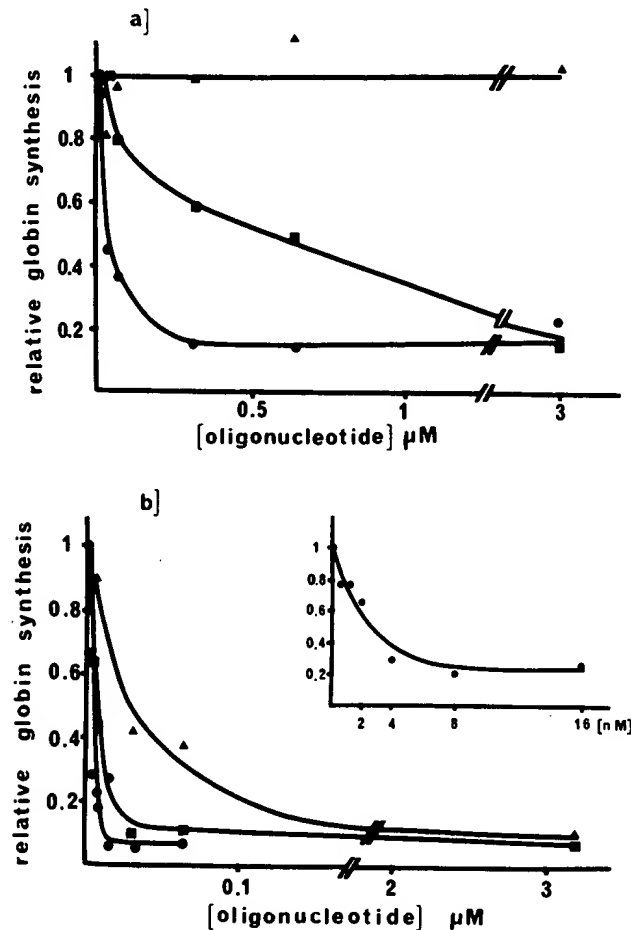


Figure 7 : Effect of 17PO- β (a) and 17PS (b) on the synthesis of rabbit β -globin in micro-injected *Xenopus* oocytes. Globin synthesis was determined from densitometer tracings of autoradiographs, relatively to the synthesis observed in the absence of added oligodeoxynucleotide. Oligonucleotides injected 6 hours prior to (▲), 6 hours after (■) or coinjected with the mRNA (●). Inset in panel (b) is an enlargement of the curve (●) in the main figure.

range had to be used to observe 50% inhibition (Figure 3). This is probably due to the weak affinity of this oligomer for its target as indicated by a low value of the melting temperature of the DNA-RNA duplex (Table 1). It is worth mentioning that the unmodified 11PO had no effect at any concentration up to 20 μ M (8).

The differences in inhibition efficiencies between the various oligomers could be related to their affinity for their target or to the sensitivity to RNase-H of the hybrid they formed with β -globin mRNA. In particular it was of interest to understand why the α -oligomer had no

effect on translation. To delineate this we first investigated the binding of 17-mers to the mRNA and then the sensitivity of oligonucleotide-mRNA hybrids to RNase-H.

Thermal stability

We compared the affinity of rabbit globin mRNA for various oligonucleotides, namely 17PO- β , 17PS, 17PO- α and 16PO- α using hybridization experiments (see Materials and Methods). The two α -oligomers have the same target but whereas 16PO- α was designed to bind RNA in an antiparallel orientation, 17PO- α was synthesized to bind in a parallel orientation (Figure 1). We did not detect non-specific interactions between oligomers and the filters. All oligonucleotides but one, 16PO- α , gave a signal from thermal elution of filter-bound complexes (data not shown). Binding of 17PO- α to the immobilized mRNA indicated that this α -oligodeoxynucleotide formed a double-stranded structure with RNA in which the two chains run parallel to each other, in good agreement with a previous report (27). Assuming ΔH values are similar for all three oligonucleotides, relative affinities of 17-mer analogues for globin mRNA can be deduced from the relative temperatures T_c of half-dissociation of the complexes, given in Table 1. Even though T_c obtained with 17PO- α was lower than that of its β -homolog it was still higher than that of 17PS. Binding of 17PO- α occurred specifically to its target region of β -globin mRNA as demonstrated by a competition experiment : translation inhibition of β -globin mRNA by 17PO- β was reversed by addition of an excess of 17PO- α both in wheat germ extracts and in *Xenopus* oocytes, indicating that the two oligomers competed for binding to the same RNA sequence (data not shown). Therefore the failure to inhibit rabbit β -globin mRNA translation with the α -derivative cannot be ascribed to a weak stability of the α -oligonucleotide/mRNA hybrids.

RNase-H activity on oligodeoxynucleotide-RNA hybrids

It was shown that RNase-H, which cleaves the RNA part of RNA-DNA hybrids, amplified the antisense effect produced by oligodeoxynucleotides both in wheat germ extracts and in *Xenopus* oocytes (6-9). We therefore investigated the activity of RNase-H on RNA associated with various complementary 17-mers (17PO- β , 17PO- α and 17PS). Rabbit globin mRNA was incubated, in the presence of the oligomers, with *E.coli* RNase-H. Aliquots of the mixtures were withdrawn at various times, spotted onto nylon membranes and probed with ^{32}P end-labelled 17PO- β . As shown on figure 8 no RNase activity was detected during the time course of the experiment in the absence of added oligonucleotide. On the other hand the presence of the various 17-mers did not prevent binding of the probe. Under our experimental conditions about 85% of the β -globin mRNA was cleaved by RNase-H after a 2 h incubation in the presence of 17PO- β . Under the same conditions, 50% mRNA remained intact in the presence of 17PS, whereas no degradation was detected with 17PO- α . Therefore, although the α -oligonucleotide was bound to its target, the hybrid was not recognized as a substrate by *E.coli* RNase-H. In contrast the phosphorothioate analogue was able to induce the cleavage of the complementary RNA as previously observed with homooligomers (12).

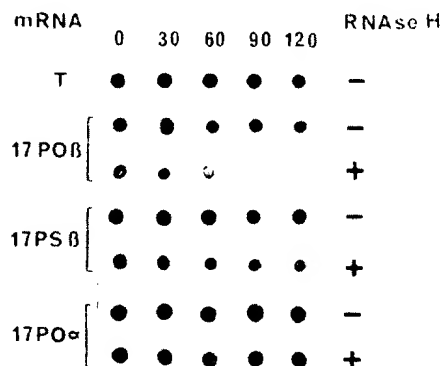


Figure 8 : Oligonucleotide-induced cleavage of rabbit β-globin mRNA by RNase-H. Dot-blot of globin mRNA incubated in the absence (T) or in the presence of 17-mers listed on the left side, in the absence (-) or in the presence of *E.coli* RNase-H (+), during the time indicated (in minutes) at the top of the autoradiograph. The blot was probed with ³²P-5'-end-labelled 17PO-β.

DISCUSSION

As part of our studies on the use of oligodeoxynucleotides as specific inhibitors of gene expression, we have chosen to examine modified oligomers with respect to their ability to inhibit protein translation. We have focused on two modifications, phosphorothioate DNA and α-DNA which render oligonucleotides resistant to nucleases, and have compared these with the normal analogues.

The expected inhibition of globin synthesis in the reticulocyte lysate system and in the wheat germ extract was observed with normal antisense oligomers in agreement with previous reports (8, 28, 42, 43). By contrast, the situation with modified oligonucleotides was more complicated. In wheat germ extract no effect on β-globin synthesis was detected in the presence of α-oligomers (either a parallel 17-mer or an antiparallel 16-mer), targeted to the coding region of the β-globin mRNA, even at high concentrations (>30 μM). The same results were obtained in *Xenopus* oocytes: none of the two α-oligodeoxynucleotides inhibited translation of microinjected rabbit globin mRNA. This anti-β-globin sequence was synthesized in both orientations because, although it has been known for some time that α-DNA forms parallel-stranded structure with β-DNA (19-23), it was more recently reported that α-dT₈, linked to a phenanthroline-copper complex, binds to polyrA in the antiparallel orientation (25). Our studies with filter-immobilized globin mRNA showed that only the parallel 17PO-α hybridized to rabbit globin mRNA, in fair agreement with a recent work using two other mRNA species (27). Therefore it seems possible that the orientation of the two strands in an α-DNA/β-RNA hybrid depends on the base sequence of the α-oligodeoxynucleotide or that

α -oligothymidylates represent a unique case where the two strands are antiparallel rather than parallel.

The absence of inhibitory effect of the parallel 17PO- α is clearly due to the lack of activity of RNase-H on the hybrid formed with β -globin mRNA. Preliminary investigations performed with a methylphosphonate derivative of the 17-mer complementary to the region 113-129 of the β -globin mRNA support this conclusion. This phosphonate oligomer (a gift from Dr. Zon) did not prevent β -globin synthesis neither in the wheat germ cell-free system nor in micro-injected *Xenopus* oocytes at any concentration (up to 50 μ M). This methylphosphonate 17-mer failed to induce the degradation of the target mRNA by the *E. coli* RNase-H.

Such an RNase-H activity was previously shown to be present both in wheat germ extracts and in microinjected oocytes (8). Both the normal 17PO and its phosphorothioate analogue 17PS induced the RNaseH activity and were strongly inhibitory in both systems. But hybrids formed by rabbit β -globin with 17PS were not more susceptible to RNase-H than the ones formed with the PO analogue in contrast to what was observed with oligo(dT)'s (12). This could arise either from differences in hybrid structures or from enzyme specificity. Since RNase-H appears to be an important component in translation inhibition by complementary oligodeoxynucleotides it might be of interest to target A-rich sequences by phosphorothioate oligomers in order to take full advantage of mRNA degradation.

The experiments with phosphorothioate oligomers in microinjected *Xenopus* oocytes confirm what was surmised from cell-free experiments. The nuclease resistance properties of the S-oligonucleotides make them very active even if microinjected long before the message (Table 1). As a consequence of both DNase resistance and RNase-H activation, nearly complete inhibition could be achieved at concentrations in the low nanomolar range, i.e. at a stoichiometry of about one oligonucleotide per four mRNA molecules indicating a catalytic effect for this oligonucleotide. Results obtained from competition experiments between 17PO- α on the one hand and 17PO- β or 17PS on the other hand are also relevant : inhibition of β -globin translation by either of the latter two oligomers can be reduced by co-injection of 17PO- α . But the concentration of the 17PO- α competitor required to get a 50% reduction of the inhibitory effect in the presence of 17PO- β was lower than that in the presence of 17PS (data not shown). This could be ascribed to the intracellular degradation of 17PO- β by DNases which results in a decreasing concentration of active antimessenger during the time course of the translation experiment. These conclusions with respect to inhibition of translation are summarized in Table 2.

However there are still unanswered questions. For example 17PS binds more weakly to globin RNA than 17PO (Table 1) and it is less active at inducing cleavage of mRNA by *E. coli* RNase-H (Figure 8). In wheat germ extracts 17PO and 17PS are equally active at inhibiting β -globin synthesis. In microinjected oocytes 17PS is about ten times more active than in the

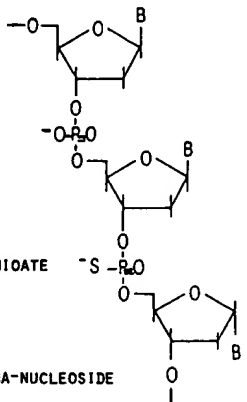
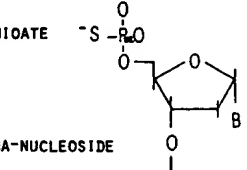
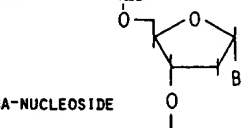
	PHOSPHODIESTER	PHOSPHOROTHIOATE	ALPHA-NUCLEOSIDE
			
RESISTANCE TO DNASES	NO	YES	YES
ACTIVATION OF RNASE H	YES	YES	NO
INHIBITION OF TRANSLATION	YES	YES	NO

Table 2 : Properties of unmodified, phosphorothioate and alpha-antisense oligodeoxynucleotides.

cell-free system whereas 17PO has quite similar activity. Therefore there might be other factors than nuclease resistance, hybrid stability and RNase-H susceptibility which are playing a role in the efficacy of oligodeoxynucleotides at inhibiting protein synthesis. One such factor could be different compartmentalization of the two oligomers inside oocytes. The kinetics of RNase-H cleavage could also be different in the two systems.

At least at low concentrations ($<1\mu\text{M}$ for 17PS) phosphorothioate analogues fulfill the criteria of anti-sense specificity: 17PS inhibits the synthesis of its target only, i.e., β -globin. But in this paper, we have demonstrated that these derivatives may be non-sequence specific inhibitors of protein synthesis if the concentration is not optimized. We have shown that this effect is highly concentration and length dependent: in wheat germ extract a 5-mer PS was not toxic at $100\mu\text{M}$ but a 15-mer containing two blocks of 5 contiguous phosphorothioates behaved like a 10-mer PS. In contrast, in the reticulocyte lysate, a 15-mer phosphodiester capped with phosphorothioate units at both ends exhibited specific behavior close to that of the all-phosphodiester analogue. The results presented in this paper can be discussed with respect to the effects of S-oligomers in other systems. For instance studies on HIV replication and protein expression have revealed two distinct mechanisms of inhibition by phosphorothioate oligodeoxynucleotides: one sequence (antisense) specific, the other non-sequence specific (15, 44, Matsukura et al., unpublished results). Kinetic studies of cellular uptake of fluorescent oligomers have identified an 80kD protein as a possible cell surface receptor for oligonucleotides and related substances (45, 46). In addition, Zhang et al. (unpublished results), have shown that the 80kD protein binds phosphorothioate with greater avidity than normal DNA. A similar observation was noted for HIV reverse transcriptase, which also binds phosphorothioate DNA with higher affinity than its oxygen congener (50). In subsequent

experiments, it has been shown that although both phosphorothioate oligomers and their normal counterparts bind to ribosomes, the former is not displaceable (Stein and Neckers, unpublished results). It is thus of interest to note that when 17PS was injected into *Xenopus* oocytes in high enough concentration (16 μ M), total protein synthesis was abolished and the oocytes exhibited altered pigmentation and then underwent extensive cytolysis. These observations may account for the non-specific cellular cytotoxicity observed when cells are exposed to concentrations of phosphorothioate DNA above 25-50 μ M for extended times. However: it is worth noting that in *Xenopus* oocytes specific translation inhibition by a 17-mer was achieved in the nanomolar range i.e. at concentrations three orders of magnitude lower than that at which toxic effects took place.

From the standpoint of antisense strategy, phosphorothioate DNA appears to be one promising member of the class of modified oligonucleotides. These compounds will very likely be alternative tools to unmodified derivatives in the field of developmental biology and in drug research. These compounds could allow one to specifically ablate the expression of a gene in cells at very low concentrations of antisense molecules, without detrimental effect or interference with the intracellular nucleotide pool, in contrast to recent reports in which unmodified phosphodiester oligonucleotides have been used (47-49).

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Abbreviations:

ODN, oligodeoxynucleotide; PO, phosphodiester oligomer; PS, phosphorothioate oligomer;
 α , alpha oligomer.

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RNase H cleavage of RNA hybridized to oligonucleotides containing methylphosphonate, phosphorothioate and phosphodiester bonds

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ABSTRACT

Three types of 14-mer oligonucleotides were hybridized to human β -globin pre-mRNA and the resultant duplexes were tested for susceptibility to cleavage by RNase H from *E. coli* or from HeLa cell nuclear extract. The oligonucleotides contained normal deoxynucleotides, phosphorothioate analogs alternating with normal deoxynucleotides, or one to six methylphosphonate deoxynucleosides. Duplexes formed with deoxyoligonucleotides or phosphorothioate analogs were susceptible to cleavage by RNase H from both sources, whereas a duplex formed with an oligonucleotide containing six methylphosphonate deoxynucleosides alternating with normal deoxynucleotides was resistant. Susceptibility to cleavage by RNase H increased parallel to a reduction in the number of methylphosphonate residues in the oligonucleotide.

Stability of the oligonucleotides in the nuclear extract from HeLa cells was also tested. Whereas deoxyoligonucleotides were rapidly degraded, oligonucleotides containing alternating methylphosphonate residues remained unchanged after 70 minutes of incubation. Other oligonucleotides exhibited intermediate stability.

INTRODUCTION

Antisense oligonucleotides are increasingly used as modulators of cellular and viral gene expression (see ref. 1-4 for review). Three classes of oligonucleotides have been used in recent investigations: antisense deoxyoligonucleotides (D-oligos), their modified counterparts and antisense RNA. All three classes have been effective in inhibiting expression of specific genes. For example, in their pioneering work Zamecnik and Stephenson (5) showed that D-oligos complementary to a segment of reiterated terminal sequence of Rous sarcoma virus inhibit viral replication. More recently phosphorothioate deoxyoligonucleosides (S-oligos, developed by Eckstein and coworkers, see ref. 6 for review), methylphosphonate deoxyoligonucleosides (MP-oligos, developed by Miller, Ts'o and coworkers, reviewed in ref. 7) as well as D-oligos have been shown to inhibit replication of the human immunodeficiency virus when they were complementary to essential viral sequences (8-11). Other modified oligonucleotides that inhibit expression of specific genes include phosphoroamidate oligonucleosides (9), α -oligonucleotides (12,13), and polylysine (14), psoralen (15) and acridine conjugated oligonucleotides (16,17). The discovery that the expression of some procaryotic genes is controlled in vivo by endogenous antisense RNA (reviewed in 18 and 19), and that expression of thymidine kinase can be inhibited by antisense RNA transcribed from a recombinant expression vector (20,21) showed that antisense RNA may also be useful in inhibiting the expression of specific genes. This early work led to a number of subsequent studies extensively reviewed in ref. 18.

Most of the reports discussed above focused on the final effect of antisense oligonucleotides on gene expression without detailed studies of the mechanism of inhibition. The two most likely mechanisms of inhibition appear to be 1) direct blocking in pre-mRNA and/or mRNA of sequences important for processing or translation and 2) degradation of the RNA transcript by RNase H at the site of oligonucleotide binding. RNase H cleaves the RNA component of RNA:DNA hybrids and is abundant in the cytoplasm and nucleus of a large number of organisms (22). Recent reports show that cleavage of RNA:DNA duplexes by RNase H was predominantly responsible for the inhibitory activity of D-oligos in several experimental systems (23-25). However, only limited data are available regarding the mechanism of action of modified oligonucleotides (3,26,27). In addition, discrepancies exist concerning the effects of antisense molecules. For example, antisense MP-oligos were found inhibitory in some but not all systems (10,11, 26,28,29) and a 1000 fold excess of antisense RNA did not inhibit the activity of chloramphenicol acetyl transferase expressed in transfected CV1 cells (30). These observations suggest that more detailed investigations are needed to discern the mechanism of inhibition by antisense oligonucleotides.

Since there are indications that RNA:MP-oligo duplexes are resistant to RNase H (3,26) we decided to study in more detail the effect of incorporation of methylphosphonate deoxynucleosides into D-oligos on the susceptibility of pre-mRNA:DNA duplexes to RNase H cleavage. To this end we have used a series of 14-mer oligonucleotides substituted with one to six methylphosphonate deoxynucleosides. For comparison, we have also tested a 14-mer D-oligo and a 14-mer S-oligo containing alternating deoxynucleotides and phosphorothioate analogs. We found that D-oligo and an alternating S-oligo form duplexes with pre-mRNA that are cleaved by *E.coli* RNase H and by the RNase H present in a crude nuclear extract from HeLa cells. In contrast, RNA in duplexes formed with an MP-oligo containing six methylphosphonate deoxynucleosides alternating with deoxynucleotides is resistant to cleavage by RNase H from both sources but becomes susceptible if the number of methylphosphonate deoxynucleosides in the oligonucleotide is decreased.

MATERIALS AND METHODS

Oligonucleotide synthesis. Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems) using standard β -cyanoethyl cycles (31). Materials for synthesis were supplied by Applied Biosystems or American Bionetics. D-oligos were deblocked and cleaved from the column with following the Applied Biosystems protocol. S-oligos were obtained in a purified form from Dr. Scott Eadie (Applied Biosystems). MP-oligos were cleaved from the solid support column by ethylenediamine: absolute ethanol (1:1 v/v) treatment at 55°C for 55 minutes, eluted with absolute ethanol followed by ethanol-water (1:1 v/v), lyophilized and resuspended in water. O.D.₂₆₀ was measured for each sample and the oligonucleotides were used in this form for all experiments. When necessary, all oligonucleotides were end labeled with [³²P] γ -labeled ATP using T4 polynucleotide kinase in 100 mM Tris pH 7.5, 20 mM MgCl₂, 10 mM DTT, 0.2 mM spermidine, 0.2 mM EDTA at 37°C for 30 minutes as recommended by the supplier (New England Biolabs). **Pre-mRNA transcription.** The DNA plasmid containing the human β -globin gene cloned under the control of the SP6 promoter (pSP64Hb Δ 6, see ref. 32), was digested with the restriction endonuclease Bam HI and transcribed and capped in vitro as described (33) using SP6 polymerase and [³²P] labeled GTP (New England Nuclear). This produced a human β -globin pre-mRNA truncated at the 3' end of the second exon.

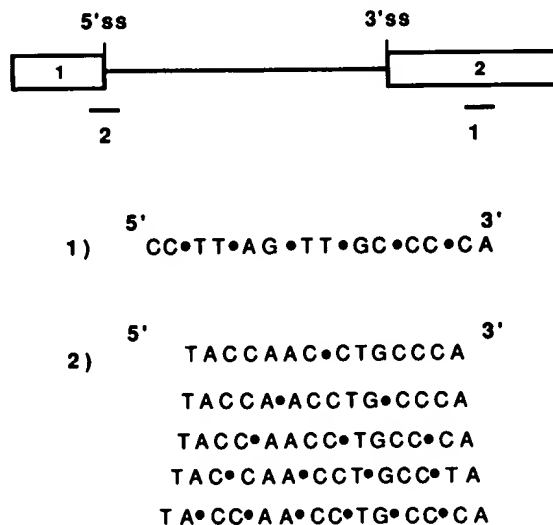


Figure 1. Target RNA and antisense oligonucleotides. The structure of the truncated human β -globin pre-mRNA used for hybridization with the oligonucleotides is shown. Exons (boxes) intron (thin line) and splice sites (5' and 3' ss) are indicated. The transcript is terminated at a Bam HI site close to the 3' end of the second exon. The positions where oligos #1 and #2 hybridize to the pre-mRNA as well as the sequence of the oligos are also shown. Dots in the oligonucleotide sequence show the position of the methylphosphonate or phosphorothioate internucleotide bonds in MP- and S-oligos. Oligo #1 was used as a normal deoxyoligonucleotide (D.1), an alternating phosphorothioate (S.1) and an alternating methylphosphonate (MP.1). Oligo #2 was used as a deoxyoligonucleotide (D.2) or containing an increasing number of methylphosphonate residues per molecule (1MP.2–6MP.2).

RNase H cleavage. E.coli RNase H was obtained from Bethesda Research Laboratories. Nuclear extract from HeLa cells (32,34) was used as a source of eucaryotic RNase H. Oligonucleotides (25 pmoles) and [32 P] labeled pre-mRNA (10 pmoles) were hybridized in vitro at 40°C for 10 minutes in 10 μ l of the following reaction mixture: for cleavage by E. coli RNase H, the hybridization was performed in 130 mM ammonium chloride; for cleavage by RNase H from HeLa cell nuclear extract, the hybridization mixture contained 12.5 mM ATP, 8.25 mM MgCl₂, 50 mM creatine phosphate and 6.5% polyvinyl alcohol. Following hybridization, for the E.coli enzyme, the reaction was performed at 37°C for 30 minutes in a total volume of 20 μ l containing 130 mM ammonium chloride, 10 mM Tris pH 7.5, 10 mM magnesium acetate, 5% sucrose and 1 μ l of RNase H. For the HeLa enzyme, 15 μ l of the nuclear extract was added and the reaction was performed at 30°C for 15 minutes. The extract contributed several buffer components so that the final concentrations of the reagents were 5 mM ATP, 3.3 mM MgCl₂, 20 mM creatine phosphate, 2.6% polyvinyl alcohol, 12.8 mM HEPES, pH 7.9, 14% glycerol, 60 mM KCl, 0.12 mM EDTA and 0.7 mM DTT, i.e., standard conditions for in vitro splicing of pre-mRNA (34). RNase H cleavage products were analyzed by electrophoresis on a 5% polyacrylamide followed by autoradiography.

Primer extension. Primer extension assay was performed in a 10 μ l reaction containing 100 mM Tris pH 7.5, 100 mM MgCl₂, 200 mM NaCl, 25 pmoles of oligonucleotide, 10 pmoles of [32 P]-pre-mRNA, 5 μ Ci of [32 P]- α -labeled dATP, 2.5 mM deoxynucleotide triphosphates and 1 μ l of AMV reverse transcriptase (Life Sciences). The primer extension

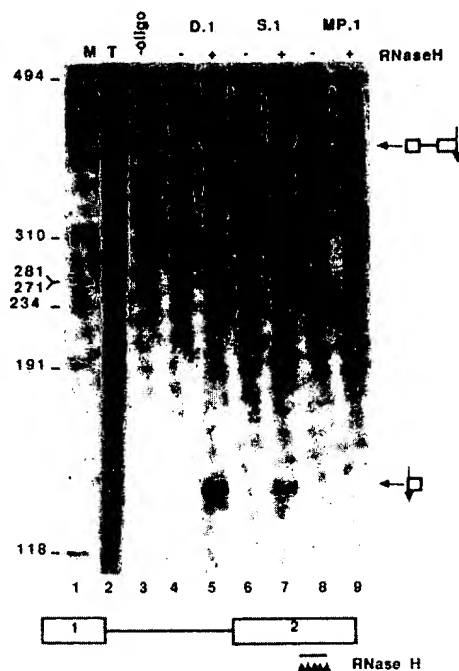


Figure 2 A. Cleavage of pre-mRNA in duplexes with D-oligo, S-oligo, or MP-oligo by RNase H from *E. coli*. [^{32}P]-pre-mRNA was hybridized with oligonucleotide # 1 (see Fig. 1) in the form of D-oligo (D.1, lanes 4 and 5), alternating S-oligo (S.1, lanes 6 and 7), or alternating MP-oligo (MP.1, lanes 8 and 9) and incubated without (-) or with (+) RNase H from *E. coli*. The resulting RNA products were separated on a 5% polyacrylamide sequencing gel. A schematic structure of RNase H cleavage products is shown at right. Below the figure, the structure of the pre-mRNA, the position of binding of oligonucleotide # 1, and cleavage by RNase H are shown. In this and subsequent figures, M (lane 1) denotes size markers (Hae III digest of ΦX174 in this figure and products of β -globin pre-mRNA splicing in the following figures), T (lane 2) denotes untreated RNA transcript (approximately 3 times the amount of radioactive RNA was loaded in this lane), -oligo (lane 3) denotes pre-mRNA mock hybridized and incubated with RNase H in the absence of oligonucleotide.

products were electrophoresed and autoradiographed as above.

Stability of oligonucleotides. Oligonucleotides, 5'-end labeled with [^{32}P], were incubated in the HeLa cell nuclear extract for 0–70 minutes in the same buffer conditions as described above and subsequently analyzed on a 20% polyacrylamide sequencing gel. The gels were autoradiographed and the amount of intact material and degradation products was quantitated by densitometry. As a measure of degradation a ratio of the amount of the intact 14-mer to a smallest degradation product, the mononucleotide, was calculated and plotted in figures 3 and 7. This way of calculation was carried out to compensate for the activity of phosphatases, which might be present in the nuclear extract and could gradually remove the radioactive label.

RESULTS

A capped fragment of human β -globin pre-mRNA (Fig. 1), obtained by transcription in vitro (see Materials and Methods), was hybridized to three types of 14-mer oligonucleotides that included a normal deoxynucleotide (D-oligo), a phosphorothioate analog (S-oligo),

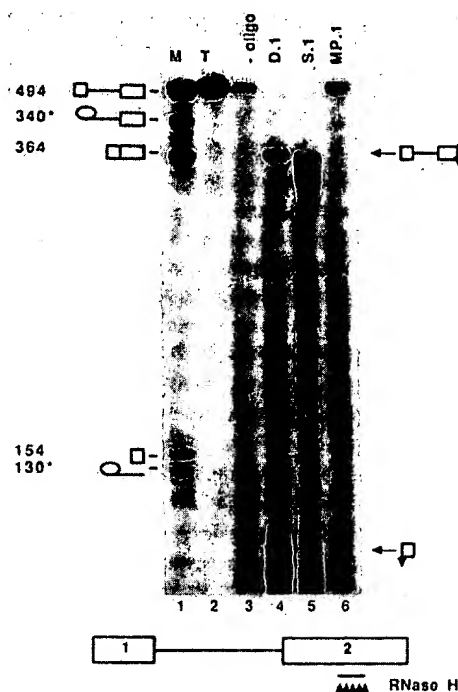


Figure 2B. Cleavage of pre-mRNA in duplexes with D-oligo, S-oligo or MP-oligo by RNase H from HeLa cell extract. The same [32 P]-pre-mRNA:oligo duplexes as in A were treated with RNase H from HeLa cell nuclear extract (lanes 4, 5, and 6). RNA products were analyzed as in A and the structure and position of the RNase H cleavage products are shown on the right. In lane 1, pre-mRNA was spliced in vitro and the resulting products used as size markers. The size and structure of the splicing products is shown at left. Asterisk indicates aberrant migration on the gel of the splicing intermediates containing lariat. Below the figure, a diagram of the RNase H site of cleavage is shown.

and a series of oligonucleotides containing an increasing number of methylphosphonate deoxynucleosides (MP-oligos). The D-oligo, the S-oligo and one of the MP-oligos were complementary to nucleotides 360–373 in the second exon of human β -globin pre-mRNA (Fig. 1, oligo 1). The S-oligo and the MP-oligo contained alternating phosphodiester and modified internucleotide bonds. An additional series of MP-oligos, which contained from one to six methylphosphonate deoxynucleosides positioned in the oligonucleotides as shown in Fig. 1, was complementary to the 5' splice site, at nucleotides 148–161 (Fig. 1, oligo 2). Substitution of six methylphosphonate deoxynucleosides resulted in an MP-oligo containing alternating methylphosphonate and phosphodiester internucleotide bonds. The duplexes formed with these oligonucleotides were tested for their susceptibility to hydrolysis by RNase H.

The pre-mRNA hybridized with the normal D-oligo, complementary to the second exon (Fig. 1, oligo 1), was incubated with E.coli RNase H and the RNA isolated from the reaction was analyzed on a 5% polyacrylamide sequencing gel. Part of the RNA was cleaved into two fragments of approximately 360 and 120 nucleotides (Fig. 2A, lane 5). Longer incubation with a larger amount of enzyme led to complete cleavage of the RNA into the same two fragments (results not shown, see also Fig. 5, lane 9). The larger cleavage product

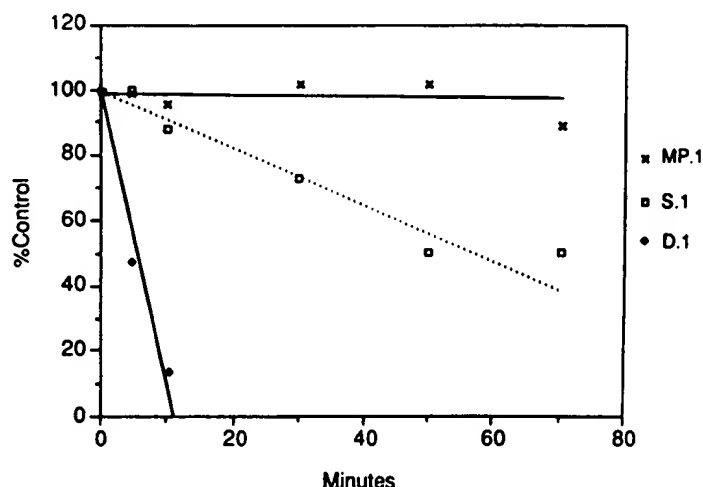


Figure 3. Stability of oligonucleotide #1 in a HeLa cell nuclear extract. D-oligo (D.1), S-oligo (S.1) and MP-oligo (MP.1) forms of oligonucleotide #1 were labeled with [32 P] using T4 polynucleotide kinase and incubated from 0–70 minutes in a HeLa cell nuclear extract. The oligonucleotides were separated on a 20% polyacrylamide sequencing gel and the amount of intact material and degradation products was quantitated by densitometry of autoradiograms. Extent of degradation was calculated as a ratio of the amount of the intact 14-mer to the smallest degradation product, the mononucleotide.

represents a capped 5' fragment of the pre-mRNA located upstream from the binding site of the oligonucleotide while the smaller fragment represents a 3' part of the RNA transcript. The mobility of the fragments on the gel is in agreement with their size predicted from the RNA sequence data. The cleavage reaction required both the oligonucleotide and the enzyme, since the RNA remained intact in control samples missing either of these components, (Fig. 2A, lanes 1 and 2, respectively). Similarly, oligonucleotide and enzyme-dependent cleavage by RNase H was also observed for a duplex containing the alternating S-oligo (Fig. 2A, lane 7) although densitometry of the film showed that the yield of the generated fragments was approximately 20 % lower than that for the D-oligo containing duplex (compare lanes 5 and 7 in Fig. 2A). In contrast, RNA hybridized under the same conditions with alternating MP-oligo was resistant to cleavage by RNase H (Fig. 2A, lane 9).

To determine whether eucaryotic RNase H would cleave these duplexes, the same samples were incubated with a nuclear extract from HeLa cells known to contain high levels of RNase H activity (32). The crude extract was used because it more closely resembles the intracellular conditions to which the duplexes would be exposed *in vivo*. Similarly as in the previous experiment, pre-mRNA in duplexes with either D-oligo or S-oligo was hydrolyzed by RNase H in the extract (Fig. 2B, lanes 4 and 5, respectively) whereas the pre-mRNA hybridized with MP-oligo remained intact (Fig. 2B, lane 6). The 360 nucleotide fragment generated by RNase H cleavage was stable in the extract and is clearly visible on a gel but the shorter 3' fragment, which was not capped, was largely degraded. The instability of the latter fragment is in agreement with the observations that the nuclear extract from HeLa cells contains a 5' to 3' exonuclease activity which is inhibited by the cap structure at the 5' end of the pre-mRNA transcript (32 and our unpublished observations).

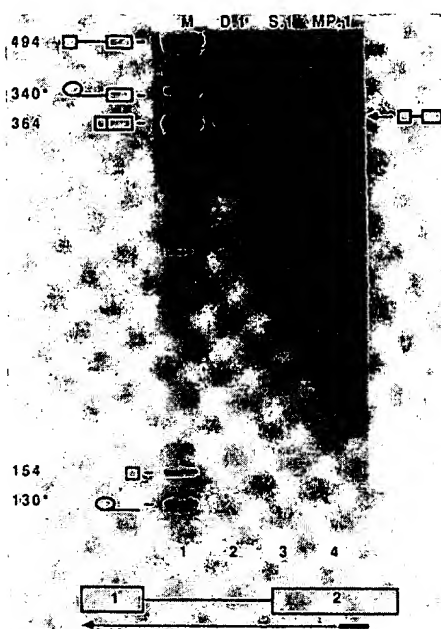


Figure 4. Primer extension analysis of oligonucleotide analogues. D-oligo, S-oligo, and MP-oligo forms of oligonucleotide #1 were hybridized to the pre-mRNA, extended with AMV reverse transcriptase and the products separated on a 5% polyacrylamide gel. The structures and positions of the primer extension products for D-oligo (D.1, lane 2), S-oligo (S.1, lane 3), and MP-oligo (MP.1, lane 4) are shown on the right. A diagram of the primer extension reaction is shown at the bottom.

The above results showed that pre-mRNA hybridized with D-oligo and an alternating S-oligo formed duplexes that were substrates for RNase H. However, the lack of cleavage of the pre-mRNA hybridized with an alternating MP-oligo either by *E. coli* RNase H or by the HeLa cell nuclear extract could have several possible explanations. Although it is likely that the pre-mRNA:MP-oligo duplex may not be a substrate for RNase H, it is also possible that 1) the alternating MP-oligo is rapidly degraded, especially in the crude nuclear extract, 2) it does not hybridize to pre-mRNA under the conditions of the experiment, or 3) it directly inhibits RNase H. A series of experiments have been performed to distinguish between these possibilities.

To test the stability of the MP-oligo in the extract, the oligo was labeled with [^{32}P] using T4 polynucleotide kinase, incubated in the nuclear extract and analyzed on a 20% polyacrylamide sequencing gel. The autoradiograms of the gel were quantitated by densitometry (see Materials and Methods). For comparison, the stabilities of D-oligo and S-oligo were also tested. Of the three oligonucleotides, MP-oligo was the most stable and remained essentially intact for at least 70 minutes of incubation. Degradation of S-oligo was also slow, with approximately 50% of the material remaining at the end of incubation whereas D-oligo was almost completely degraded after 10 minutes (Fig. 3).

To determine whether the MP-oligo is able to hybridize to the pre-mRNA, we tested it in a primer extension assay (Fig. 4). All three oligonucleotides, including MP-oligo, generated extension products of the expected length (374 nucleotides) indicating that,

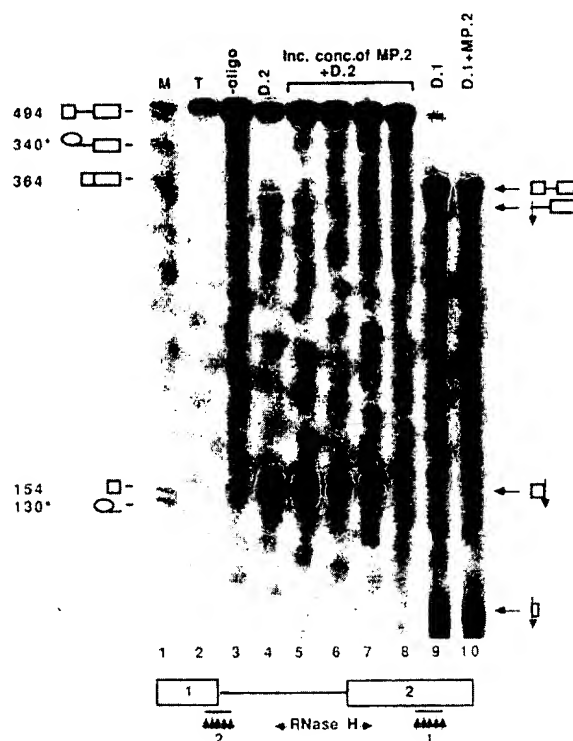


Figure 5. Competition assay using D-oligo #2, alternating MP-oligo #2 and D-oligo #1. 10 pmoles of [32 P]-labeled pre-mRNA was hybridized with 0, 0.002, 0.02, 0.2 or 10 O.D.₂₆₀ of MP-oligo #2 followed by hybridization with 0.002 O.D.₂₆₀ of D-oligo #2 and treatment with RNase H from *E. coli* (lanes 4–8, respectively). The position and structure of the RNase H cleavage products after separation on a 5% polyacrylamide gel are shown by the middle two diagrams on the right. To test for direct inhibition of RNase H by MP-oligo, pre-mRNA was hybridized without (lane 9) or with (lane 10) MP-oligo #2 followed by hybridization with D-oligo #1 and treatment with RNase H from *E. coli*. The position and structure of these RNase H cleavage products are shown by the top and bottom diagrams on the right. Below the figure is a schematic representation of the RNase H cleavage site for the oligonucleotides used above.

similarly to D-oligo and S-oligo, MP-oligo formed a duplex with the pre-mRNA. Additional faint bands seen in lane 2 result from premature termination of reverse transcription. The lower yield of extension products from the S-oligo and MP-oligo duplexes as compared to the D-oligo duplex is probably due to the lower efficiency of the AMV reverse transcriptase with the modified primers, as previously observed (35). Since S-oligo and MP-oligo yielded similar amounts of extension product, the resistance of the RNA:MP-oligo duplex to hydrolysis by RNase H (Fig. 2A, lane 9 and Fig. 2B, lane 6) was not due to the lack of hybridization of this oligonucleotide to the pre-mRNA.

To obtain additional evidence that the interaction of MP-oligo with the pre-mRNA is sequence specific and to show that the MP-oligo does not directly inhibit RNase H, we carried out a competition experiment with different D- and MP-oligos. The pre-mRNA was hybridized to increasing concentrations of the alternating MP-oligo complementary to the 5' splice site (see Fig. 1B, oligo 2) followed by incubation with a normal D-oligo

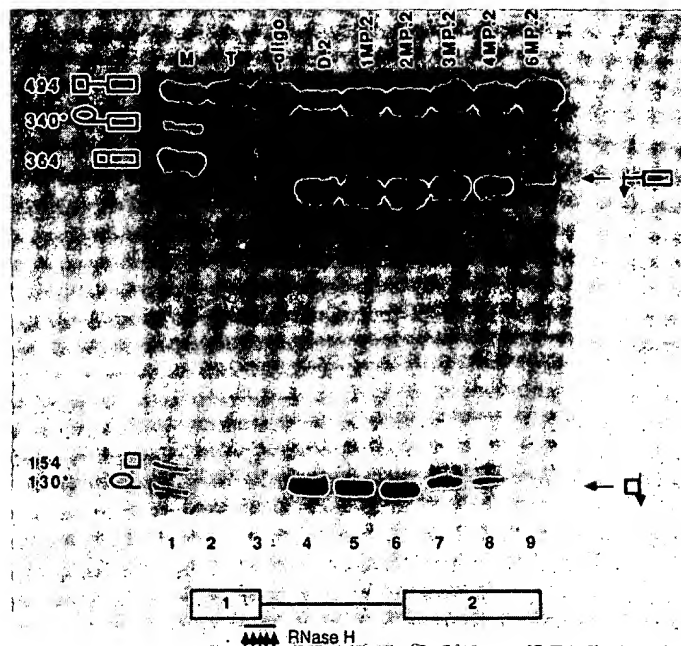


Figure 6. Effect of the number of MP-deoxynucleosides in oligonucleotide #2 on the susceptibility of RNA:MP-oligo duplexes to RNase H cleavage. Pre-mRNA:oligo duplexes were formed and treated with *E. coli* RNase H as described in Materials and Methods and analyzed on a 5% polyacrylamide sequencing gel. Size markers and controls (lanes 1–3) are as described in Fig. 2A. Lane 4, RNase H cleavage of a duplex with D-oligo #2 (D.2). Lanes 5–9, RNase H cleavage of duplexes with MP-oligos containing one to six MP-deoxynucleosides, respectively. Diagrams are as described in Fig. 2A.

of the same sequence. The duplexes were then treated with RNase H in the nuclear extract as described above. As expected, in the absence of MP-oligo, the pre-mRNA was cleaved into two RNase H cleavage products, approximately 330 and 150 nucleotides long (Fig. 5, lane 4). Cleavage with RNase H was progressively inhibited by increasing amounts of MP-oligo as indicated by the disappearance of the cleavage products in lanes 5–8. Other bands visible in these lanes represent unspecific degradation products since they are also present in the control sample incubated without either oligonucleotide (Fig. 5, lane 3). To ascertain that the MP-oligo did not inhibit RNase H directly, the D-oligo complementary to the exon sequence (Fig. 1, oligo 1) was hybridized to the pre-mRNA with or without the alternating MP-oligo complementary to the 5' splice site (Fig. 1, oligo 2). When the duplexes were incubated with the nuclear extract, RNase H cleaved the pre-mRNA:D-oligo duplex and was not inhibited in the presence of the MP-oligo (Fig. 5, lanes 9 and 10).

We conclude from the experiments that 1) the MP-oligo did not have any direct inhibitory effect on RNase H; 2) the increasing resistance of duplexes seen in lanes 5–8 must be due to competition between D-oligo and MP-oligo for the same sequence in the pre-mRNA; 3) the MP-oligo formed a sequence specific duplex with the pre-mRNA which was resistant to RNase H.

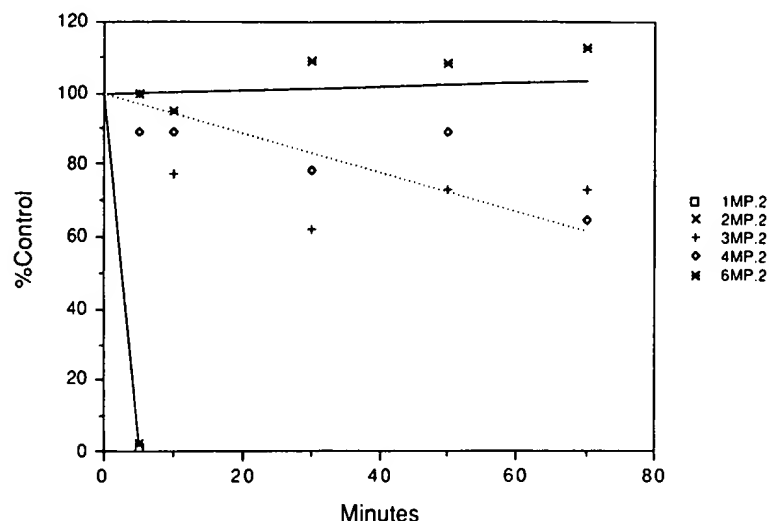


Figure 7. Effect of the number of MP-deoxynucleosides in oligonucleotide #2 on the stability of the MP-oligos in a nuclear extract from HeLa cells. MP-oligos containing one to six MP-deoxynucleosides (1MP.2–6MP.2, respectively) were incubated in the nuclear extract from HeLa cells and analyzed as described in Fig.3.

Since the above results showed that duplexes between pre-mRNA and alternating MP-oligo are resistant to RNase H we wanted determine the minimum number of methylphosphonate deoxynucleosides required to confer resistance to a duplex molecule. We have synthesized a series of 14-mer oligonucleotides, containing from one to six methylphosphonate deoxynucleosides positioned in the oligonucleotides as shown in Fig. 1 (Fig. 1, oligo 2). To ascertain that the resistance to RNase H observed above is not due to a particular sequence or secondary structure at the oligonucleotide binding site in the second exon, this series was made complementary to a different region of the pre-mRNA, at the 5' splice site. These MP-oligos were hybridized to the pre-mRNA and the duplexes were subjected to hydrolysis by *E. coli* RNase H.

RNA hybridized to MP-oligos containing one or two methylphosphonate deoxynucleosides was cleaved by RNase H almost as easily as that in the control duplex with D-oligo (Fig. 6, compare lane 4 with lanes 5 and 6). RNA in duplexes with MP-oligos which contained three, four and six methylphosphonate deoxynucleosides, i.e., in which methylphosphonate bonds were separated by three, two or one phosphodiester bond (see Fig. 1, oligo 2), was increasingly resistant to cleavage by the enzyme (Fig. 6, lanes 7–9, respectively). Interestingly, the resistance of these duplexes to RNase H hydrolysis paralleled the stability of the corresponding MP-oligos in the nuclear extract. MP-oligos containing one or two methylphosphonate deoxynucleosides were degraded rapidly and the stability of the oligonucleotides increased with an increased number of methylphosphonate deoxynucleosides in the molecule (Fig. 7)

DISCUSSION

Results presented above show that D-oligo, S-oligo and MP-oligos hybridize to pre-mRNA in a sequence specific manner. Duplexes formed with the first two types of oligonucleotides are susceptible to cleavage by RNase H from *E. coli* and from HeLa cells. This agrees

with the results of Stein et al. (27) on cleavage of polyA:phosphorothioate oligo dT duplexes by RNase H from *E. coli* and with the reports of other groups on inhibition of translation by RNase H cleavage of D-oligo containing duplexes (23–25). In contrast, duplexes formed with alternating MP-oligos are not substrates for RNase H from *E. coli* or HeLa cells. Their resistance to the enzyme decreases with the decrease in the number of methylphosphonate deoxynucleosides in the MP-oligo. Our results also show that the presence of phosphorothioate and methylphosphonate deoxynucleosides promotes the stability of the oligonucleotides in the nuclear extract from HeLa cells.

It has been shown by a number of investigators that D-oligos, S-oligos and MP-oligos can be used *in vivo* to inhibit expression of a specific gene in a sequence specific manner (reviewed in 1–4). The results presented here suggest different mechanisms of inhibition by different classes of oligonucleotides. Whereas D-oligos and S-oligos probably lead to the degradation of target RNA transcript by RNase H, MP-oligos containing only methylphosphonate deoxynucleosides or alternating MP-oligos exert their effect in a different manner, most likely by interfering with the accessibility of RNA to factors essential for processing, transport or translation. In most experiments, designed to eliminate RNA viruses, viral transcripts, or the inappropriate expression of endogenous RNA, the cleavage of an RNA:DNA hybrid is desired. In some situations, however, the lack of cleavage of the RNA can be advantageous. For example, the ability to block specific sequences in intact pre-mRNA may be very useful in studies of the interactions of the splicing factors with the pre-mRNA substrate. In fact, our recent experiments show that alternating MP-oligo complementary to the 5' splice site of the β -globin pre-mRNA will hybridize to the pre-mRNA substrate without degrading it and will inhibit splicing in the nuclear extract from HeLa cells (Furdon and Kole, unpublished).

Our results with MP-oligos containing an increasing number of methylphosphonate residues suggest that the properties of the MP-oligos may be manipulated to achieve a desired effect. Introduction of a few methylphosphonate deoxynucleosides will not inhibit the activity of RNase H but will markedly increase the stability of the resulting MP-oligos. Thus, if degradation of the target RNA is the goal of the experiment such MP-oligos should be more effective than normal D-oligos. Conversely, for blocking of specific sequences in intact RNA, MP-oligos made exclusively with methylphosphonate deoxynucleosides or with alternating methylphosphonate and normal deoxynucleotides should be preferable. The alternating MP-oligos have a higher negative charge than fully modified MP-oligos allowing for easier handling and purification on polyacrylamide gels. In addition, in alternating MP-oligos the number of diastereoisomers generated during chemical synthesis (36) is drastically reduced which may increase the effective concentration of the molecules able to hybridize to target RNA. Finally, MP-oligos that contain two phosphodiester bonds surrounded by stretches of methylphosphonate deoxynucleosides can be used as tools for precise cleavage of the target RNA by RNase H.

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Oligothymidylate Analogues Having Stereoregular, Alternating Methylphosphonate/Phosphodiester Backbones as Primers for DNA Polymerase[†]

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ABSTRACT: Oligothymidylate analogues having stereoregular, alternating methylphosphonate/phosphodiester backbones, d-Tp(TpTp)₄T isomers I and II and d-Tp(TpTp)₃T(pT)₁₋₅ isomers I and II, were prepared by methods analogous to the phosphotriester synthetic technique. The designations isomer I and isomer II refer to the configuration of the methylphosphonate linkage, which is the same throughout each isomer. Analogues with the type I methylphosphonate configuration form very stable duplexes with poly(dA) while those with the type II configuration form either 2T:1A triplexes or 1T:1A duplexes with poly(dA) of considerably lower stabilities. The oligothymidylate analogues were tested for their ability to initiate polymerizations catalyzed by *Escherichia coli* DNA polymerase I or calf thymus DNA polymerase α on a poly(dA) template. Neither d-Tp(TpTp)₄T nor d-Tp(TpTp)₃TpT served as initiators of polymerization while d-Tp(TpTp)₃T(pT)₂₋₅

showed increasing priming ability as the length of the 3'-oligothymidylate tail increased. Analogues with type I methylphosphonate configuration were more effective initiators than the type II analogues at 37 °C. The apparent activation energies of polymerizations initiated by d-Tp(TpTp)₃T(pT)_{4 and 5} isomer I were greater than those for reactions initiated by isomer II or d-(Tp)₁₁T. The results suggest that DNA polymerase interacts with the charged phosphodiester groups of the primer molecule and may help stabilize primer/template interaction. At least two contiguous phosphodiester groups are required at the 3' end of the analogue primers in order for polymerization to occur. Interactions between the polymerase and primer also appear to occur with phosphodiester groups located at sites remote from the 3'-OH polymerization site and may be influenced by the configuration of the methylphosphonate group.

Oligonucleotide analogues having nonionic alkyl phosphotriester or methylphosphonate backbones have served as models for studying the influence of backbone structure on the conformation and interactions of nucleic acids (Miller et al., 1971; Pless & Ts'o, 1977; Miller et al., 1979; Kan et al., 1980). As a result of their ability to form complexes with complementary nucleic acid sequences, these noncharged analogues have been used to probe and regulate cellular nucleic acid function both in the test tube and in living cells (Miller et al., 1974, 1977, 1981; Barrett et al., 1974; Jayaraman et al., 1981). In a recent publication we described the synthesis and physical properties of novel oligothymidylate analogues having methylphosphonate

groups of fixed configuration arranged in an alternating manner with negatively charged phosphodiester groups throughout the backbone of the oligonucleotide (Miller et al., 1980a). The structure of the analogues may be written as d-Tp(TpTp)₄T^p where *p* denotes 3'-5' methylphosphonate linkages with either *R* or *S* configuration throughout and *p* denotes 3'-5' phosphodiester linkages.

These oligothymidylate analogues are able to form complexes with both poly(dA) and poly(rA). The stoichiometries and stabilities of the complexes are dependent upon the configuration of the methylphosphonate group. Although the

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¹ Abbreviations: d-NpN, an oligonucleotide having a 3'-5' internucleoside methylphosphonate linkage; d-NpN, an oligonucleotide having a 3'-5' *p*-chlorophenyl phosphotriester linkage; MST, (mesitylenesulfonyl)tetrazole; TSNi, (*p*-toluenesulfonyl)-4-nitroimidazole; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; CD, circular dichroism. The symbols used to represent protected nucleosides and oligonucleotides follow the IUPAC-IUB Commission on Biochemical Nomenclature (1970) recommendations.

methylphosphonate linkages of the analogues are completely resistant to nuclease hydrolysis, the phosphodiester linkages either are resistant or are slowly hydrolyzed (Miller et al., 1980a). We have also found that these analogues inhibit nuclease-catalyzed hydrolysis of DNA.

The presence of negatively charged phosphodiester groups in these analogues provides potential recognition sites for various nucleic acid enzymes. This and the ability of the analogues to form complexes with complementary polynucleotides suggested that they might serve as primers or substrates for nucleic acid replication enzymes such as DNA polymerase or DNA ligase. In this paper we explore the ability of d-Tp(TpTp)₄T and oligothymidylate derivatives of this analogue, d-Tp(TpTp)₃T(pT)₁₋₅, to initiate template-directed polymerizations catalyzed by DNA polymerases from *Escherichia coli* and calf thymus. The results of these studies provide some new insights into possible recognition features between the polymerases and their oligonucleotide initiators.

Experimental Procedures

Thymidine, d-(Tp)₁₁T, poly(dA), *E. coli* DNA polymerase I, and calf thymus DNA polymerase α were purchased from P-L Biochemicals. Tritium-labeled thymidine triphosphate was obtained from New England Nuclear. Anhydrous pyridine (Miller et al., 1980b), (mesitylenesulfonyl)tetrazole (Stawinski et al., 1977), (*p*-toluenesulfonyl)-4-nitroimidazole (Gough et al., 1979), d-[(MeO)₂Tr]Tp(TpTp)₃TpCNET isomers I and II, and d-Tp(TpTp)₄T isomers I and II (Miller et al., 1980a) were prepared as previously described. Preparative thick-layer silica gel chromatography (PTLC) was performed on PLK 5 F plates (20 cm \times 20 cm \times 1 mm) (Whatman, Inc.) with mixtures of methanol and chloroform as solvents. Preparative high-performance liquid chromatography (HPLC) was performed on a Partisil M-9 silica gel column (9.0 mm \times 50 cm) (Whatman, Inc.). The column was eluted at a flow rate of 4.0 mL/min with a linear gradient of 0–20% methanol in chloroform (total volume 240 mL). Analytical and preparative ion-exchange HPLC were carried out on a Pellionex AL WAX column (4.6 \times 50 cm). The column was eluted at a flow rate of 1.0 mL/min with a linear gradient of 0.001–0.50 M or 0.001–1.0 M ammonium acetate in 60% ethanol/water (total volume 40 mL). Analytical reversed-phase HPLC was performed on a Partisil 10 ODS-3 column (4.6 mm \times 25 cm) (Whatman, Inc.). The column was eluted at a flow rate of 2.5 mL/min with a linear gradient of 0–25% acetonitrile in 0.10 M ammonium acetate buffer, pH 5.8 (total volume 50 mL).

Preparation of Oligothymidylate Methylphosphonate Analogues. The same general synthetic procedures were used as previously described for the preparation of alternating methylphosphonate/phosphodiester oligothymidylate analogues and oligonucleotide *p*-chlorophenyl phosphotriesters (Miller et al., 1980a,b). Protected oligothymidylate methylphosphonate analogues d-[(MeO)₂Tr]Tp(TpTp)₃Tp(Tp)_nTOAc isomers I and II ($n = 0-4$) were each prepared by reaction of the triethylammonium salt of d[(MeO)₂Tr]Tp(TpTp)₃Tp isomer I or isomer II with dTOAc or d-(Tp)_nTOAc ($n = 1-4$) in anhydrous pyridine in the presence of MST or TSNI. Reactions employing MST were run for 3 h while those employing TSNI were run overnight at room temperature. After workup, the protected oligomers were purified by silica gel PTLC or by preparative silica gel HPLC. The reaction conditions and yields are given in Table I.

The pure protected oligomers were treated with 1.0 mL of solution containing 0.017 M tetra-*n*-butylammonium fluoride in tetrahydrofuran/pyridine/water (8:1:1 v/v) at room tem-

perature for 24 h, followed by treatment with 2 mL of 50% concentrated ammonium hydroxide in pyridine at 4 °C for 2.5 days. After evaporation of the solvents the residue was treated with 0.5 mL of 80% acetic acid at 37 °C for 30 min. The resulting d-Tp(TpTp)₃T(pT)₁₋₅ analogues were each purified by preparative HPLC on a Pellionex AL WAX column (Leutzing et al., 1978). Fractions containing the pure oligomers were combined, and the ammonium acetate was removed by passing the oligomer solution through a DEAE-cellulose column (2.5 \times 4 cm) as previously described (Miller et al., 1980b). The oligomer was eluted from the DEAE column with 1 M ammonium bicarbonate. After removal of the buffer by evaporation, the oligomer was dissolved in 50% ethanol/water, and the solution was stored at 0 °C. For use in the physical and biological experiments, aliquots containing the required amount of oligomer were evaporated to dryness, and the oligomer was dissolved in the buffer used in the particular experiment.

The deprotected oligomers were characterized by digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase. Twelve nanomoles of oligomer was treated with 2 μ g of snake venom phosphodiesterase and 0.9 unit of bacterial alkaline phosphatase in 25 μ L of buffer containing 10 mM Tris-HCl, pH 8.0, and 2 mM magnesium chloride for 24 h at 37 °C. The digest was briefly heated at 100 °C and then analyzed by reversed-phase HPLC. The areas under the product peaks were measured, and the ratio of d-TpT/d-T was calculated (Table II).

Interactions of d-Tp(TpTp)₃T(pT)₁₋₅ with Poly(dA). Ultraviolet spectra were recorded on a Varian 219 spectrophotometer equipped with a thermostated cell compartment and temperature readout accessory. The continuous variation experiments and melting experiments were carried out as previously described (Miller et al., 1971). Melting curves were corrected for the change in absorbance of poly(dA) with temperature. No change in absorbance was observed for d-Tp(TpTp)₃T(pT)₁₋₅ over the temperature range investigated. The following molar extinction coefficients were used: poly(dA), $\Sigma_{257\text{nm}} = 9100$; d-(Tp)₁₁T, $\Sigma_{266\text{nm}} = 8700$; d-Tp(TpTp)₃T(pT)₁₋₅ isomers I and II, $\Sigma_{265\text{nm}} = 8300$ and $\Sigma_{265\text{nm}} = 8600$, respectively.

DNA Polymerase Catalyzed Reactions. Enzymatic reactions were run in duplicate under the conditions described below. The reaction mixtures were preincubated for 10 min at the appropriate temperature in the absence of enzyme. The reactions were then initiated by addition of enzyme. Aliquots (4 μ L for DNA polymerase I reactions, 6 μ L for DNA polymerase α reactions) were withdrawn at appropriate times and added to 1 mL of ice-cold 5% trichloroacetic acid solution. The solution was filtered on a glass fiber filter (Whatman GF/C). The filter was washed with four 1-mL aliquots of 2 N hydrochloric acid and four 1-mL aliquots of 95% ethanol. After being dried under a heat lamp, the filters were counted in Betafluor (National Diagnostics, Inc.) scintillation fluid.

(A) *E. coli* DNA Polymerase I Reactions. Reactions were carried out in 30 μ L of solution containing 20 mM Tris-HCl, pH 7.6, 5 mM magnesium chloride, 0.1 M potassium chloride, 0.6 μ g of bovine serum albumin, 50 μ M poly(dA) (base concentration), 5 μ M oligothymidylate (base concentration), and 100–200 μ M ³H-labeled thymidine triphosphate, sp act. 250–500 mCi/mmol. Reactions were initiated by addition of 0.125–0.25 unit of DNA polymerase I.

(B) Calf Thymus DNA Polymerase α Reactions. Reactions were carried out in 40 μ L of solution containing 20 mM potassium phosphate, pH 7.2, 6 μ g of bovine serum albumin, 1

Table I: Synthesis of Protected Oligothymidylate Methylphosphonates

oligomer	isomer	d-[(MeO) ₂ Tr]- Tp(TpTp) _n TP (μ mol)	5'-OH component (μ mol)	condensing agent (μ mol)	yield (%)
d-[(MeO) ₂ Tr]Tp(TpTp) ₁ TPTOAc	I	5.0	d-TOAc (7.5)	MST (30)	37
d-[(MeO) ₂ Tr]Tp(TpTp) ₂ TPTPTOAc	I	5.0	d-TpTOAc (7.5)	MST (30)	48
	II	2.5	d-TpTOAc (3.8)	MST (15)	7
d-[(MeO) ₂ Tr]Tp(TpTp) ₃ T(pT) ₁ TOAc	I	5.0	d-(Tp) ₂ TOAc (7.5)	MST (30)	51
	II	2.5	d-(Tp) ₂ TOAc (3.8)	MST (15)	7
d-[(MeO) ₂ Tr]Tp(TpTp) ₄ T(pT) ₂ TOAc	I	2.5	d-(Tp) ₃ TOAc (3.8)	TSNI (15)	13
	II	2.5	d-(Tp) ₃ TOAc (3.8)	TSNI (15)	9
d-[(MeO) ₂ Tr]Tp(TpTp) ₅ T(pT) ₃ TOAc	I	5.0	d-(Tp) ₄ TOAc (7.5)	TSNI (20)	50
	II	5.0	d-(Tp) ₄ TOAc (7.5)	TSNI (20)	40

Table II: Characterization of Oligothymidylate Methylphosphonates

oligomer	isomer	elution position on Pellionex AL WAX ^a	enzymatic hydrolysis products ^b (dTpT/dT)	
			obsd	theory
d-Tp(TpTp) ₁ TP	isomer I	0.21 M	3.7/1.0	4.0/1.0
d-Tp(TpTp) ₂ T(pT) ₁	isomer I	0.27 M	1.9/1.0	2.0/1.0
	isomer II	0.26 M	1.9/1.0	
d-Tp(TpTp) ₃ T(pT) ₂	isomer I	0.36 M	1.4/1.0	1.3/1.0
	isomer II	0.34 M	1.2/1.0	
d-Tp(TpTp) ₄ T(pT) ₃	isomer I	0.43 M	0.8/1.0	1.0/1.0
	isomer II	0.43 M	0.9/1.0	
d-Tp(TpTp) ₅ T(pT) ₄	isomer I	0.56 M	0.6/1.0	0.8/1.0
	isomer II	0.56 M	0.7/1.0	

^a Ammonium acetate concentration at which oligomer is eluted from a Pellionex AL WAX column. ^b Conditions for hydrolysis are given under Experimental Procedures.

mM dithiothreitol, 6 mM magnesium acetate, 50 μ M poly(dA) (base concentration), 5 μ M oligothymidylate (base concentration), and 100 μ M ³H-labeled thymidine triphosphate, sp act. 250–500 mCi/mmol. Reactions were initiated by addition of 0.6–1.1 units of DNA polymerase α .

Results

Synthesis of Oligothymidylate Methylphosphonates. The syntheses of d-Tp(TpTp)_nT isomers I and II have been previously described (Miller et al., 1980a), and the preparations of the oligothymidylate derivatives, d-Tp(TpTp)_nT(pT)_m ($n = 1-5$), closely follow these procedures. The reaction conditions

and isolated yields are given in Table I. The deprotected oligomers were purified by high-performance liquid chromatography and were characterized by hydrolysis with a combination of snake venom phosphodiesterase and bacterial alkaline phosphatase as indicated in Table II. The designations isomer I and isomer II refer to the configuration of the methylphosphonate linkage, which is the same throughout each isomer. The absolute configuration of the type I and type II methylphosphonate group has not as yet been determined.

Interaction of Oligothymidylate Methylphosphonates with Poly(deoxyadenylic acid). Complex formation between the oligothymidylate methylphosphonates and poly(dA) was determined under the salt conditions used in the DNA polymerase I and DNA polymerase α experiments (see the following section). The stoichiometries, melting temperatures, and temperatures at which the complexes are melted are summarized in Table III for the DNA polymerase I ionic strength conditions. Similar results were obtained for complexes in the DNA polymerase α buffer, although the melting temperatures were approximately 2 °C lower (data not shown). The melting profiles of all the complexes are sigmoidal in shape and show the expected increase in T_m as the oligomer chain length increases.

Ability of Oligothymidylate Methylphosphonates To Serve as Primers for DNA Polymerase. Table IV compares the initial rates of polymerization reactions initiated by d-Tp(TpTp)_nT and d-Tp(TpTp)_nT(pT)_m ($n = 1-5$) with those initiated by d-(Tp)_nT on a poly(dA) template. The reactions were carried out at 37 °C by using either *E. coli* DNA polymerase I or calf thymus DNA polymerase α and were monitored by following the incorporation of ³H-labeled thy-

Table III: Complex Formation between Oligothymidylate Methylphosphonate Analogues and Poly(dA)^a

oligomer	isomer	stoichiometry of complex	T_m (°C)	temp at which complex is completely melted (°C)
d-(Tp) ₁ T		1T:1A	36.5	47
d-Tp(TpTp) ₁ T	isomer I	1T:1A	32.4	40
	isomer II	2T:1A	2 ^b	15 ^b
d-Tp(TpTp) ₂ TP	isomer I	1T:1A	31.4	39
	isomer II	1T:1A	33.5	40
d-Tp(TpTp) ₃ T(pT) ₁	isomer I	2T:1A	8.0	20
	isomer II	1T:1A	36.5	49
d-Tp(TpTp) ₄ T(pT) ₂	isomer I	mixture of 1T:1A and 2T:1A	13.5	25
	isomer II	1T:1A	39.0	50
d-Tp(TpTp) ₅ T(pT) ₃	isomer I	1T:1A	19.0	31
	isomer II	1T:1A	41.8	52
			24.2	34

^a Buffer: 20 mM Tris-HCl (pH 7.2), 5 mM magnesium chloride, and 0.1 M potassium chloride; total nucleotide concentration 2.5×10^{-5} M. ^b Buffer: 0.1 M sodium cocodylate (pH 6.8); total nucleotide concentration 2.5×10^{-5} M.

Table IV: Ability of Oligothymidylate Methylphosphonate Analogues To Initiate DNA Polymerase Catalyzed Polymerization on a Poly(dA) Template at 37 °C

primer	initial rate relative to d-(Tp) ₁₁ T-initiated reaction ^a			
	DNA polymerase I		DNA polymerase α	
	isomer I	isomer II	isomer I	isomer II
d-(Tp) ₁₁ T	1.000		1.000	
d-Tp(TpTp) ₄ T	0.000	0.000	0.000	0.000
d-Tp(TpTp) ₃ TpT	0.000		0.000	
d-Tp(TpTp) ₃ T(pT) ₂	0.011	0.000	0.006	0.004
d-Tp(TpTp) ₃ T(pT) ₃	0.120	0.000	0.149	0.015
d-Tp(TpTp) ₃ T(pT) ₄	0.535	0.047	0.437	0.072
d-Tp(TpTp) ₃ T(pT) ₅	0.984	0.331	0.721	0.229

^a The reaction conditions are given under Experimental Procedures.

midylic acid into trichloroacetic acid precipitable material. The initial incorporation of label was linear with time for reactions catalyzed by either enzyme. No incorporation of label was observed in the absence of either primer or template molecules.

Neither d-Tp(TpTp)₄T nor d-Tp(TpTp)₃TpT initiated polymerization on the poly(dA) template in the presence of DNA polymerase I. Polymerization was observed for reactions initiated by d-Tp(TpTp)₃T(pT)₂₋₅ isomer I. The initial rates of polymerization increased as the chain length of the oligothymidylate tail of the analogue increased. The rate of polymerization initiated by d-Tp(TpTp)₃T(pT)₅ isomer I was almost identical with that of the reaction initiated by d-(Tp)₁₁T at 37 °C. In contrast, d-Tp(TpTp)₃T(pT)_n isomer II served as a primer only when *n* was 4 or 5. Although the initial rates of the reactions primed by the shorter oligomers were considerably less than that for the reaction initiated by d-(Tp)₁₁T, the extent of these polymerizations approached that of d-(Tp)₁₁T after prolonged incubation.

Similar results were obtained for reactions catalyzed by calf thymus DNA polymerase α. No priming activity was observed for d-Tp(TpTp)₄T or d-Tp(TpTp)₃TpT, while d-Tp(TpTp)₃T(pT)₂₋₅ showed increasing priming ability as the chain length of the oligothymidylate tail increased. In contrast to the results obtained with DNA polymerase I, the d-Tp(TpTp)₃T(pT)_{2 and 3} isomer II analogues each served as primers in the DNA polymerase α catalyzed reactions. Although the initial rates of these reactions were quite small, prolonged incubation resulted in polymerization levels comparable to those obtained in the d-(Tp)₁₁T/poly(dA) control reactions. On the other hand, no polymerization was observed, even after prolonged incubation, when DNA polymerase I was the catalyst.

The effects of temperature on polymerization reactions initiated by d-(Tp)₁₁T and d-Tp(TpTp)₃T(pT)_{4 and 5} were determined. The results of a typical experiment are shown in Figure 1 for polymerizations catalyzed by DNA polymerase I with d-(Tp)₁₁T and d-Tp(TpTp)₃T(pT)₄ as primers. The data are presented in the form of an Arrhenius plot. The apparent activation energies calculated from these plots are given in Table V.

The rates of the DNA polymerase I catalyzed polymerization primed by d-Tp(TpTp)₃T(pT)₄ are very temperature dependent. The rate of the isomer I primed reaction diminishes rapidly with decreasing temperature and is barely detectable below 19 °C. In contrast, the isomer II primed reaction has a rate maximum at 27 °C. At low temperatures, isomer II is a better primer than is isomer I. The rates of polymeriza-

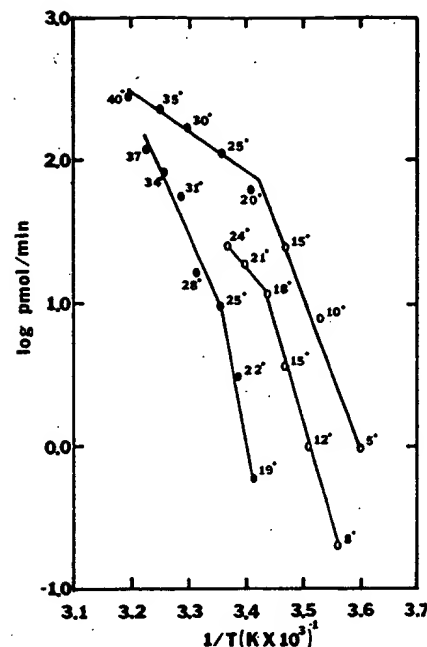


FIGURE 1: Arrhenius plot of the effect of temperature on the rate of *E. coli* DNA polymerase I catalyzed polymerizations on a poly(dA) template using d-(Tp)₁₁T (○) and d-Tp(TpTp)₃T(pT)₄ isomer I (●) and isomer II (○) as primers. The reaction conditions are given under Experimental Procedures.

Table V: Apparent Activation Energies for Reactions Catalyzed by DNA Polymerase I on a Poly(dA) Template^a

primer	<i>E</i> _{act,app} (kcal/mol) ^b
d-(Tp) ₁₁ T	44.5 (5-20)
d-Tp(TpTp) ₃ T(pT) ₄ isomer I	11.9 (20-35)
isomer II	91.5 (19-25)
	32.7 (25-35)
	66.9 (8-18)
	21.5 (18-24)
d-Tp(TpTp) ₃ T(pT) ₅ isomer I	56.7 (5-20)
isomer II	23.8 (20-35)
	38.9 (5-25)

^a The reaction conditions are given under Experimental Procedures. ^b The apparent activation energies were determined from Arrhenius plots similar to Figure 1 and are the average values obtained from at least two experiments. The temperature range (degree Celsius) is in parentheses.

tions initiated by d-Tp(TpTp)₃T(pT)₅ isomer I increased over the temperature range 5–35 °C, while isomer II showed a rate maximum at approximately 25 °C. Similar temperature dependence was observed for reactions catalyzed by DNA polymerase α.

The apparent activation energies of DNA polymerase I catalyzed polymerizations initiated by d-Tp(TpTp)₃T(pT)₄ isomers I and II and d-Tp(TpTp)₃T(pT)₅ isomer I are between 1.3 and 2.7 times greater than those observed for d-(Tp)₁₁T-initiated polymerization (Table V). The apparent activation energy for the d-Tp(TpTp)₃T(pT)₅ isomer II initiated reaction is similar to that of the d-(Tp)₁₁T-initiated reaction over the temperature range 5–25 °C. Similar results were obtained for the DNA polymerase α catalyzed reactions.

Discussion

We have examined the ability of d-Tp(TpTp)₄T and a series of 3'-oligothymidylate derivatives of this analogue, d-Tp(TpTp)₃T(pT)₁₋₅, to initiate polymerizations catalyzed by DNA polymerase on a poly(dA) template. The oligothymidylate derivatives were prepared by methods analogous

to the phosphotriester approach used to synthesize oligodeoxyribonucleotides (Miller et al., 1980b). Thus, 3'-*O*-acetylthymidine or 3'-*O*-acetyloligothymidylate *p*-chlorophenyl phosphotriesters, d-(Tp)_{*n*}TOAc (*n* = 1-4), were added to either the all *R* or all *S* methylphosphonate isomers of d-[(MeO)₂Tr]Tp(TpTp)_{*n*}Tp (Miller et al., 1980a). As shown in Table I, reactions carried out on a 5-μmol scale generally proceeded in satisfactory yield, while the yields for the 2.5 μmol scale reactions were quite low. These low yields can be attributed to the difficulty of excluding small traces of moisture from the condensation reaction mixtures.

The *p*-chlorophenyl phosphate protecting groups were selectively removed with tetra-*n*-butylammonium fluoride in aqueous tetrahydrofuran (Ogilvie & Beaucage, 1979), a reagent which does not attack methylphosphonate linkages (Miller et al., 1980a). The percentage of unwanted internucleotide phosphotriester bond cleavage increased as the chain length of the oligomers increased. However, the use of pyridine aldoximate, a reagent which does not cause internucleotide phosphotriester bond cleavage (Recse et al., 1978), was precluded since this reagent hydrolyzes methylphosphonate linkages.

The deprotected oligonucleotides were completely hydrolyzed to d-T and d-TpT by a combination of snake venom phosphodiesterase and bacterial alkaline phosphatase. The expected ratios of products were obtained for each analogue (Table II). Although snake venom phosphodiesterase alone hydrolyzed the oligothymidylate tail of d-Tp(TpTp)_{*n*}T(pT)_{*n*}, further hydrolysis of the remaining d-Tp(TpTp)_{*n*}T was incomplete even after prolonged incubation. Previous studies have shown that this enzyme completely hydrolyzes the phosphodiester but not the methylphosphonate linkages of d-Tp(TpTp)_{*n*}T in an exonucleolytic manner starting at the 3' end of the analogue (Miller et al., 1980a). The inability of snake venom phosphodiesterase to completely hydrolyze the methylphosphonate-flanked phosphodiester bonds of d-Tp(TpTp)_{*n*}T(pT)_{*n*} suggests that the d-pT initially formed in the reaction serves as an inhibitor of hydrolysis of these phosphodiester bonds.

The analogues d-Tp(TpTp)_{*n*}T(pT)_{*n*} with the type I methylphosphonate configuration each formed 1T:1A duplexes with poly(dA) (Table III). Two ionic strength conditions were chosen for these experiments: 0.1 M potassium chloride, 20 mM Tris-HCl, and 20 mM magnesium chloride, the buffer used for the DNA polymerase I reactions, and 20 mM potassium phosphate and 6 mM magnesium acetate, the buffer used for the DNA polymerase α reactions. The stabilities of the duplexes in the DNA polymerase I buffer system were slightly greater than those measured in the DNA polymerase α buffer system and reflect the greater ionic strength of this buffer. As expected, the *T_m* values of the duplexes increased as the chain length of the analogues increased.

In contrast to the behavior of the analogues with type I methylphosphonate configuration, d-Tp(TpTp)_{*n*}T(pT)_{*n*} isomer II forms either 2T:1A triplexes or 1T:1A duplexes with poly(dA). The type of complex formed depends upon the length of the 3'-terminal oligothymidylate tail. The *T_m* values of the isomer II/poly(dA) complexes are 17-30 °C lower than those of the isomer I/poly(dA) duplexes. This behavior is similar to that previously observed for d-Tp(TpTp)_{*n*}T isomer I vs. d-Tp(TpTp)_{*n*}T isomer II. The influence of the methylphosphonate configuration on the stoichiometry and the stability of the analogue/poly(dA) complexes may reflect differences in the hydration of the methylphosphonate/phosphodiester backbone of the oligomer in the duplex vs. the

single-strand forms (Miller et al., 1980a).

The ability of the oligonucleoside methylphosphonates to serve as initiators of polymerization catalyzed by either *E. coli* DNA polymerase I or calf thymus DNA polymerase α on a poly(dA) template was examined. The initial rates of polymerization observed are compared with those observed for d-(Tp)₁₁T-initiated polymerizations in Table IV. Neither d-Tp(TpTp)_{*n*}T nor d-Tp(TpTp)_{*n*}TpT served as primers for the polymerization reactions, even after prolonged (24-h) incubation. The presence of two thymidylate residues at the 3' end of the analogue resulted in priming activity by d-Tp(TpTp)_{*n*}T(pT)_{*n*} isomer I. Further additions of 3'-thymidylate residues gave increasingly more efficient primers. Thus the initial rate of polymerization initiated by d-Tp(TpTp)_{*n*}T(pT)_{*n*} isomer I was nearly the same as that of d-(Tp)₁₁T-initiated polymerization. In contrast to the results obtained with isomer I, isomer II showed considerably less ability to initiate polymerization at 37 °C.

The results suggest that the presence of methylphosphonate linkages in the backbone of the primer molecule can significantly perturb the priming ability of the oligonucleotides. Since all the oligomers tested with the type I methylphosphonate configuration form stable duplexes with poly(dA), the lack of priming ability does not result from inability of the oligomer to bind to the template. This observation suggests that the presence of the methylphosphonate groups may perturb the binding of the polymerase to the initiator/template complex. Thus binding sites on the polymerase may interact directly with the charged phosphodiester backbone of the oligonucleotide primer. This conclusion is in agreement with recent studies by Fisher & Korn (1981) on the effects of magnesium ion concentration on polymerization reactions. Their results suggest that phosphodiester groups of the primer interact via a magnesium complex with binding sites of KB cell DNA polymerase α. Introduction of nonionic methylphosphonate groups could thus directly eliminate binding interactions between the primer backbone and the enzyme or could perturb the interactions of adjacent phosphodiester groups with the enzyme.

Previous studies have shown that a free 3'-hydroxyl group is required for initiation of polymerization (Huberman & Kornberg, 1970). NMR investigations have suggested that the zinc atom associated with *E. coli* DNA polymerase I binds to the 3'-hydroxyl group of the primer molecule (Springgate et al., 1973). The observation that d-Tp(TpTp)_{*n*}T(pT)_{*n*} isomer I but not d-Tp(TpTp)_{*n*}TpT isomer I can initiate polymerization suggests that at least two contiguous phosphodiester groups are required in molecules of this type for polymerization to occur. These phosphodiesters could serve in addition to the 3'-hydroxyl group as binding points between the polymerase and the 3' end of the primer molecule.

In the case of the analogues with the type II methylphosphonate backbone configuration, only d-Tp(TpTp)_{*n*}T(pT)_{*n*} isomers, which form 1T:1A duplexes with poly(dA), are able to prime polymerization reactions catalyzed by DNA polymerase I at 37 °C. However, the results shown in Table III indicate that at this temperature the duplexes should be completely melted out. These observations therefore suggest that complex formation between the initiator and the template is not required in order for initiation of polymerization to occur. Thus, the polymerase may stabilize binding of the initiator with the template. Similar suggestions have been made for replication of single-strand DNA by T4 phage DNA polymerase (Goulian et al., 1968), for replication of homopolymers by calf thymus DNA polymerase α (Cassani & Bollum, 1969; Chang

et al., 1972), and for replication of single-stranded "hook" type primer/templates by KB cell DNA polymerase α (Fisher & Korn, 1979).

Results obtained with calf thymus DNA polymerase α were similar to those of the DNA polymerase I experiments. In this case, however, d-Tp(TpTp)₃T(pT)₂ and ₃ isomers II, which form 2T:1A triplexes with poly(dA), can also initiate polymerization. Possibly in this system the polymerase selectively stabilizes duplex formation between the analogues and the template.

The effects of temperature on polymerization reactions initiated by d-(Tp)₁₁T and d-Tp(TpTp)₃T(pT)₄ and ₅ were examined (Figure 1 and Table V). Arrhenius plots of the rate vs. temperature data were biphasic with a break point occurring between 20 and 25 °C. This behavior is in contrast to the linear plot observed for the polymeric primer/template system poly(d-A-T) whose apparent activation energy is 17 kcal/mol over the temperature range 4–40 °C (McClure & Jovin, 1975). The biphasic nature of the plots for the oligonucleotide-primed polymerizations may result from a change in the rate-determining step of the polymerization reaction as the temperature is increased.

For reactions catalyzed by DNA polymerase I, the order of apparent activation energies for the oligonucleotide-initiated reactions is d-Tp(TpTp)₃T(pT)₄ > d-Tp(TpTp)₃T(pT)₅ > d-(Tp)₁₁T. Both d-Tp(TpTp)₃T(pT)₄ isomers I and II and d-Tp(TpTp)₃T(pT)₅ isomers I and II form 1T:1A duplexes with poly(dA). The CD spectra of these duplexes are virtually identical with that of the d-(Tp)₁₁T/poly(dA) duplex (data not shown). This suggests that the conformation of the analogue/poly(dA) duplexes are very similar if not identical with that of d-(Tp)₁₁T/poly(dA). Thus it is unlikely that the increased apparent activation energies of the analogue-initiated polymerizations vs. that of the d-(Tp)₁₁T-initiated polymerization are due to differences between the conformation of the analogue/poly(dA) duplexes and that of the d-(Tp)₁₁T/poly(dA) duplex.

The increased apparent activation energies could be a consequence of unfavorable interactions between the polymerase and the methylphosphonate groups of the analogue primer backbone. Such altered interactions could influence the rate of polymerization by directly affecting the formation of the enzyme/primer/template complex. Alternatively, the rates of subsequent steps in the polymerization process, such as translocation of the polymerase, could be altered by unfavorable interactions. The methylphosphonate groups in d-Tp(TpTp)₃T(pT)₄ and ₅ are located some distance removed from the site of the polymerization reaction. Thus interactions between the polymerase and the initiator backbone may extend up to at least five nucleotide units from the 3'-OH terminus. This would be consistent with the model of Fisher & Korn, which suggests that up to seven phosphodiester linkages may be involved in primer/polymerase binding. The observation that the apparent activation energies of the type I analogues are greater than those of the type II analogues suggests that such interactions may be influenced by the configuration of adjacent methylphosphonate groups.

The results presented in this paper suggest that DNA polymerase interacts with charged phosphodiester groups of the backbone of the primer molecule. Such interactions appear

to occur with phosphodiester groups located at the 3' terminus of the primer as well as with groups located at sites remote from the 3' end. Although d-Tp(TpTp)T isomer I does not serve as an initiator of DNA polymerization, preliminary experiments have shown that it does inhibit d-(Tp)₁₁T-initiated polymerization on a poly(dA) template catalyzed by DNA polymerase α . Further characterization of this inhibitory reaction may provide additional insights into the interactions of DNA polymerase with primer/template complexes.

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Oligothymidylate Analogues Having Stereoregular, Alternating Methylphosphonate/Phosphodiester Backbones.

SYNTHESIS AND PHYSICAL STUDIES*

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Two decathymidylate analogues, d-(TpTp),TpT-isomer 1 and isomer 2, having stereoregular, alternating methylphosphonate/phosphodiester backbones were prepared. The phosphodiester linkages of d-(TpTp),TpT are cleaved slowly by snake venom phosphodiesterase in a stepwise manner, while slow random cleavage occurs with micrococcal nuclease which hydrolyzes isomer 2 faster than isomer 1. The CD spectra of isomer 1 and d-(Tp),T are identical suggesting they have similar conformations, while that of isomer 2 shows an overall reduction of $[\theta]$. Isomer 1 forms a 1T·1A complex with poly(dA) and both 1T·1A and 2T·1A complexes with poly(rA), while isomer 2 forms a 2T·1A complex of low thermal stability with poly(dA) and no complex with poly(rA). The T_m values of the partially nonionic d-(TpTp),TpT·polynucleotide complexes are less dependent on salt concentration than are those of d-(Tp),T. The stoichiometry and CD spectra of the complexes suggest that poly(dA)·isomer 1 duplex assumes a B-type geometry while isomer 2·poly(dA)·isomer 2 triplex and the isomer 1·poly(rA) complexes have an A-type geometry. Although there are no apparent differences between steric restrictions to rotation about the backbones of either isomer 1 or 2, or steric restrictions to complex formation, the results suggest that the configuration of the methylphosphate linkage controls: 1) interaction with nucleases, 2) oligomer conformation, and 3) interaction with polynucleotides. The latter effects may result from differences in solvation of the two isomers.

have been used as probes of nucleic acid sequence-function relationships *in vitro* (6, 7) and have been shown to affect biochemical processes in living cells in culture (8).

Studies on dideoxyribonucleoside methylphosphonates indicate that the configuration of the methylphosphonate linkage influences the conformation of the dimer and its interactions with complementary polynucleotides (3, 4). The nonionic phosphonate linkage appears to be resistant to nuclease hydrolysis and also enables the dimers to penetrate cellular membranes. The unique physical properties of these dimers suggested that longer oligonucleotides containing methylphosphonate linkages might have novel properties as substrates for nucleic acid enzymes. In order to provide materials for enzymatic studies and to explore further the effect of methylphosphonate stereochemistry on oligomer conformation and interactions, we have prepared the decathymidylate analogues shown in Fig. 1. These analogues have stereoregular, alternating methylphosphonate-phosphodiester internucleotide linkages. In this paper, we describe the synthesis of these analogues, physical studies on their conformation and interactions with complementary polynucleotides, and some preliminary studies on their interactions with nuclease enzymes.

MATERIALS AND METHODS

Thymidine, d-(Tp),T, poly(dA), and poly(rA) were purchased from P-L Biochemicals. The dipyrindinium salt of methylphosphonic acid (3), mesitylenesulfonyl tetrazolide (3), 5'-O-dimethoxytritylthymidine (9), 3'-O- β -benzoylpropionylthymidine (10), 5'-O-dimethoxytritylthymidine-3'-methylphosphonate (3), the β -cyanoethyl ester of thymidine-3'-methylphosphonate (3), and *p*-chlorophenylphosphorodichloridate (11) were prepared according to literature procedures. Thin layer silica gel chromatography (TLC) was performed on E. Merck Silica Gel 60 F₂₅₄ plastic-backed sheets (0.2 mm thick); preparative thick layer silica gel chromatography was performed on PLK 5 F plates (20 cm \times 20 cm \times 1 mm) (Whatman Inc.) and preparative silica gel column chromatography was carried out at atmospheric pressure in glass columns packed with Merck Silica Gel 60 (70 to 230 mesh). Analytical silica gel high pressure liquid chromatography (HPLC) was carried out on a column (2.1 mm \times 1 m) packed with HC Pellozil (Whatman, Inc.). The column was eluted at a flow rate of 1 ml/min with a 40-ml linear gradient of chloroform to 20% methanol in chloroform. Analytical reversed phase HPLC was performed on a Partisil ODS-2 column (4.6 mm \times 25 cm) (Whatman, Inc.). The column was eluted at a flow rate of 2.5 ml with the following gradient: 10% acetonitrile for 5 min, followed by a linear gradient (5 to 20 min) of 10% to 20% acetonitrile in 0.10 M ammonium acetate buffer, pH 5.8. Preparative paper chromatography was carried out on Whatman No. 3MM paper using Solvent F: 1-propanol/concentrated ammonium hydroxide/water (55:10:35, v/v).

Preparation of d-[(MeO)₂Tr]TpTO β B-d-[(MeO)₂Tr]TpCE (5.4 g, 8 mmol) was treated with a solution containing 69 ml of pyridine, 23 ml of water, and 23 ml of distilled triethylamine overnight at room temperature. The solvents were evaporated and the residue was co-

Oligonucleotides having modified sugar phosphate backbones serve as important models of nucleic acids. Nonionic deoxyribooligonucleotide alkyl phosphotriesters, d-Np(Et)N, and dideoxyribonucleoside methylphosphonates, d-NpN, have provided information on oligonucleotide conformation in solution (1-4) and the effect of electrostatic charge on nucleic acid interactions (5). In addition, nonionic oligonucleotides

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The abbreviations used are: d-Np(Et)N, an oligonucleotide ethyl phosphotriester; d-NpN, oligonucleotide having a 3'-5' internucleoside methyl phosphonate linkage; d-NpN, an oligonucleotide having a 3'-5' *p*-chlorophenyl phosphotriester linkage; MST, mesitylenesulfonyl tetrazolide. The symbols used to represent protected oligonucleotides follow the IUPAC-IUB Commission on Biochemical Nomenclature recommendations (1970).

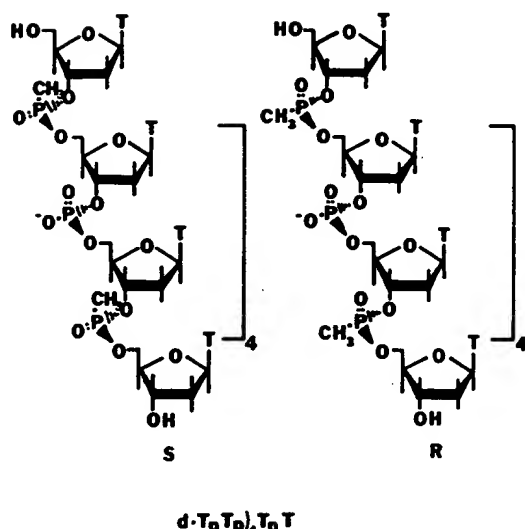


FIG. 1. Decathymidylate diastereoisomers containing stereoregular, alternating methylphosphonate phosphodiester internucleotide bonds.

evaporated with two (20 ml) portions of pyridine. 3'-O- β -Benzoylpropionyl thymidine (4.82 g, 12 mmol) was added; the nucleosides were dried by evaporation with pyridine and the residue, after solution in 40 ml of pyridine, was treated with 3.04 g (12 mmol) of mesitylene-sulfonyl tetrazolidine. After 2½ h an additional 3.04 g of MST were added and incubation was continued for 3 h. The reaction mixture was diluted with 20 ml of ice-cold 50% aqueous pyridine. The solution was poured into 400 ml of 5% sodium bicarbonate and extracted with two (250 ml) portions of chloroform. After drying over anhydrous sodium sulfate and concentration, the chloroform extract was chromatographed on silica gel (3.5 × 48 cm) using ethyl acetate (1.5 liters) and ethyl acetate/tetrahydrofuran (1:1, 2 liters) as solvents. Fractions containing pure isomer 1 (first to elute from column), pure isomer 2 and isomers 1 and 2 were pooled separately. The mixed fractions were rechromatographed on silica gel columns which were eluted with 30% tetrahydrofuran in ethyl acetate. Each isomer was dissolved in a small volume of tetrahydrofuran and the solutions were added to a 100-fold excess of hexane with stirring. The resulting precipitates were collected by filtration, washed with hexane, and dried under vacuum. The following amounts were obtained: isomer 1, 2.98 g (2.96 mmol, 37%); isomer 2, 1.96 g (1.95 mmol, 24.5%); isomers 1 and 2, 0.15 g (0.149 mmol, 1.9%). The UV spectral properties and the silica gel TLC and HPLC mobilities are given in Table II. Removal of the protecting groups from each dimer gave d-TpT-1 and d-TpT-2 whose pmr spectra were identical with previously prepared material (3).

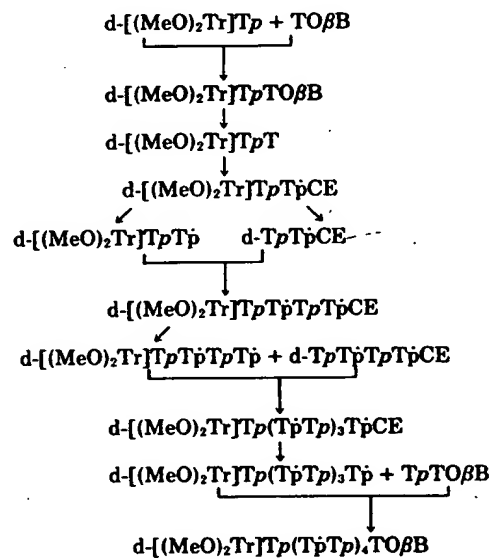
Preparation of d-[(MeO)₂Tr]TpT—A solution of d-[(MeO)₂Tr]TpTO β B (isomer 1, 2.13 g, 2.12 mmol; isomer 2, 1.84 g, 1.83 mmol) in 20% acetic acid/pyridine (isomer 1, 16 ml; isomer 2, 14 ml) was treated with hydrazine hydrate (isomer 1, 0.51 ml; isomer 2, 0.44 ml) overnight at room temperature. Each reaction mixture was diluted with 100 ml of 5% sodium bicarbonate and extracted with two (80 ml) portions of chloroform which were combined and dried over anhydrous sodium sulfate. After concentration, each chloroform extract was chromatographed on silica gel (3.5 × 35 cm) which was eluted with 500 ml of chloroform, 500 ml of 4% methanol in chloroform, and 500 ml of 10% methanol in chloroform. The following amounts of d-[(MeO)₂Tr]TpT were obtained after precipitation from hexane: isomer 1, 1.43 g (1.69 mmol, 80%); isomer 2, 0.87 g (1.03 mmol, 56%). The UV spectral properties and the silica gel TLC and HPLC mobilities of each dimer are given in Table II.

Preparation of d-[(MeO)₂Tr]TpTpCE—The reaction was carried out in a glove bag filled with dry nitrogen. Dry d-[(MeO)₂Tr]TpT (isomer 1, 1.43 g, 1.69 mmol; isomer 2, 0.87 g, 1.03 mmol) was dissolved in pyridine (isomer 1, 3.4 ml; isomer 2, 2.0 ml). Dry benzenesulfonic acid (isomer 1 reaction, 1.19 g, 6.76 mmol; isomer 2 reaction, 0.70 g, 4 mmol) dissolved in anhydrous pyridine (isomer 1 reaction, 5.0 ml; isomer 2 reaction, 3.0 ml) was treated with anhydrous triethylamine (isomer 1 reaction, 6.8 mmol; isomer 2 reaction, 4 mmol). Triazole (isomer 1 reaction, 0.35 g, 5.1 mmol; isomer 2 reaction, 0.21 g, 3.0 mmol) dissolved in dry tetrahydrofuran (isomer 1, 17 ml; isomer 2, 10 ml) was treated sequentially with triethylamine (isomer 1, 0.71 ml,

5.1 mmol; isomer 2, 0.42 ml, 3.0 mmol) and *p*-chlorophenylphosphorodichloridate (isomer 1, 0.37 ml, 2.54 mmol; isomer 2, 0.22 ml, 1.5 mmol). After 10 min the solution was filtered and the pyridine solution of d-[(MeO)₂Tr]TpT was added to the filtrate. After 1 h, the triethylammonium salt of benzenesulfonic acid was added followed by addition of dry hydrazylonitrile (isomer 1, 0.24 ml, 3.4 mmol; isomer 2, 0.14 ml, 2 mmol). The reaction mixture was concentrated to 6 ml and incubated for 1 h at room temperature and 18 h at 4°C. The reaction mixture was poured into 150 ml of ice-cold 5% sodium bicarbonate, extracted with two (100 ml) portions of chloroform and dried over anhydrous sodium sulfate. Each extract was chromatographed on silica gel (isomer 1, 3 × 34 cm; isomer 2, 2.5 × 35 cm) using chloroform (isomer 1, 750 ml; isomer 2, 500 ml) and with 5% methanol in chloroform (isomer 1, 1 liter; isomer 2, 1 liter). The following amounts of d-[(MeO)₂Tr]TpTpCE were obtained after precipitation from hexane: isomer 1, 1.31 g (1.22 mmol, 72%); isomer 2, 0.74 g (0.69 mmol, 67%). The UV spectral properties and chromatographic mobilities of the dimers are given in Table II.

Preparation of Protected Oligonucleotides—The dimethoxytrityl group was removed from protected oligonucleotides by treatment of the oligonucleotide (1 mmol) dissolved in 3.7 ml of methanol with 15 ml of 80% acetic acid for 2 h at 37°C. The detritylated oligonucleotide was isolated by precipitation from hexane. The cyanoethyl group was removed from oligomers terminating in a 3'-*p*-chlorophenyl- β -cyanoethylphosphotriester group by treating the oligomer (1 mmol) with a solution containing 8.5 ml of pyridine, 2.9 ml of water, and 2.9 ml of triethylamine for 1 h at room temperature followed by evaporation of the solvents. Each condensation reaction was run for 3½ h at room temperature following the general procedures described for d-[(MeO)₂Tr]TpTO β B except only one portion of MST was used. After workup, the oligomers were purified by silica gel column chromatography or by preparative thick layer chromatography and were isolated by precipitation from hexane. The specific reaction conditions and the yields obtained are given in Table I. The UV spectral properties and the chromatographic mobilities of the fully protected oligomers are given in Table II.

Preparation of d-(TpTp)₂TpT—d-[(MeO)₂Tr]Tp(TpTp)₂TO β B (1.7 to 3.0 μ mol) was stirred with 12 μ l of hydrazine hydrate in 0.40 ml of 20% acetic acid/pyridine solution for 18 h at room temperature. The residue obtained after evaporation of the solvents was treated with 1.0 ml of a solution containing 0.017 M tetrabutylammonium fluoride in tetrahydrofuran/pyridine/water (8:1:1, v/v) at room temperature for 24 h, and was then treated with 2 ml of 50% concentrated ammonium hydroxide in pyridine at 4°C overnight. The solvents were evaporated and the residue was treated with 0.5 ml of 80% acetic acid at 37°C for 30 min. The resulting d-(TpTp)₂TpT was purified by paper chromatography (isomer 1, *R_F* 0.35; isomer 2, *R_F* 0.35; d-pT, *R_F* 0.42). The UV spectral properties and HPLC mobilities of d-(TpTp)₂TpT are given in Table II. The molar extinction coefficients of each isomer were determined as follows. The UV spectrum of each isomer was measured in water at pH 6.3. The oligomer was then digested to monomers and dimers by treatment with 0.1 N NaOH at



SCHEME 1

60°C for 20 h, and the quantity of thymidine was determined spectrophotometrically using $\epsilon_{267} = 7.38 \times 10^3$ for thymidine at pH 13.

Physical Studies on d-(TpTp),TpT and Their Interactions with Polynucleotides—Ultraviolet spectra were recorded on a Varian 219 equipped with a thermostated cell compartment and temperature readout accessory. Circular dichroism spectra were recorded on a Cary 60 spectropolarimeter in a thermostated cell. The continuous variation experiments, melting experiments, and circular dichroism experiments were carried out as previously described (1). The melting curves were corrected for the change in absorbance of poly(dA) and poly(rA) with temperature. No change in absorbance was observed for d-(Tp)₃T or d-(TpTp),TpT over the temperature range investigated. The following molar extinction coefficients were used: poly(rA), $\epsilon_{257 \text{ nm}} = 10,000$; poly(dA), $\epsilon_{257} = 9,100$ (5); d-(Tp)₃T, $\epsilon_{266 \text{ nm}} = 8,700$ (12); d-(TpTp),TpT-1, $\epsilon_{265} = 8,300$; d-(TpTp),TpT-2, $\epsilon_{265} = 8,600$.

Treatment of d-(TpTp),TpT with Nuclease Enzymes—Enzymatic reactions were run using the conditions described below. Aliquots (10 μ l) of the digests were withdrawn at various time intervals, briefly heated at 70°C, and examined by reverse phase HPLC on Partisil OBS-2. Spleen phosphodiesterase: 22 nmol of oligonucleotide were incubated with 4 μ g of enzyme in 108 μ l of buffer containing 0.1 M ammonium acetate, pH 6.2. Snake venom phosphodiesterase: 11 nmol of oligonucleotide were incubated with 2 μ g of enzyme in 55 μ l of buffer containing 10 mM Tris·HCl, pH 8.0, 2 mM magnesium chloride. S₁ nuclease: 22 nmol of oligonucleotide were incubated with 100 units of enzyme in 90 μ l of buffer containing 0.05 M sodium chloride, 0.05 M ammonium acetate, pH 5.0, 1 mM zinc acetate. Micrococcal nuclease: 11 nmol of oligonucleotide were incubated with 24 units of

enzyme in 45 μ l of buffer containing 0.029 M sodium borate, pH 8.8, 0.0014 M calcium chloride.

RESULTS

Synthesis of the Decathymidylate Analogues—The synthetic procedure used to prepare the decathymidylate analogues, d-(TpTp),TpT, is shown in Scheme 1. The synthesis involves first preparation of the fully protected dimer, d-[(MeO)₂Tr]TpTO β B. The two diastereoisomers were separated by silica gel column chromatography and are designated as isomer 1 and isomer 2 in order of their elution from the column.

Each pure diastereoisomer was then used to prepare longer protected oligomers containing methylphosphonate linkages of the same configuration. These oligomers are designated as isomer 1 or isomer 2 to indicate from which dimer they were synthesized. The β -benzoylpropionyl group was removed selectively by treatment of the dimer with hydrazine hydrate (10). The 3'-hydroxyl group of the resulting d-[(MeO)₂Tr]TpT was then phosphorylated with the *bis*-triazolide derivative of *p*-chlorophenyl phosphate followed by esterification with hydroacrylonitrile to give d-[(MeO)₂Tr]TpTpCE. The fully protected tetramer and octamer, d-[(MeO)₂Tr]Tp(TpTp)_nTpCE ($n = 1, 3$) were then prepared by condensation of appropriately

TABLE I
Preparation of protected oligothymidylates

3'-Phosphoryl component		5'-OH component	MST	Product	Yield
mmol		mmol	mmol	mmol	%
d-[(MeO) ₂ Tr]TpTp		d-TpTpCE		d-[(MeO) ₂ Tr]TpTpTpTpCE	
Isomer 1	0.62	0.50	2.24	0.32	65
Isomer 2	0.35	0.27	1.40	0.19	66
d-[(MeO) ₂ Tr]TpTpTpTp		d-TpTpTpTpCE		d-[(MeO) ₂ Tr]Tp(TpTp) ₃ TpCE	
Isomer 1	0.147	0.117	0.59	0.077	66
Isomer 2	0.087	0.070	0.35	0.055	79
d-[(MeO) ₂ Tr]Tp(TpTp) ₃ Tp		d-TpTO β B		d-[(MeO) ₂ Tr]Tp(TpTp) ₄ TO β B	
Isomer 1	0.005	0.0075	0.03	0.0028	56
Isomer 2	0.005	0.0075	0.03	0.0017	34

TABLE II
Spectral properties and chromatographic mobilities of oligodeoxythymidylates

Oligomer	Isomer	Spectral properties ^a			Silica gel TLC ^b				HPLC ^c retention time
		λ_{max} nm	λ_{min} nm	$\epsilon_{235/260}$	5%	10%	15%	20%	
d-[(MeO) ₂ Tr]TpTO β B	1	237 265 (sh)	227	1.66	0.08	0.33			8.9
	2	237 265 (sh)	227	1.63	0.03	0.24			10.9
d-[(MeO) ₂ Tr]TpT	1	235 267	230 250	1.36	0.01	0.18		0.54	14.0
	2	235 267	230 250	1.35	0.01	0.17		0.52	13.8
d-[(MeO) ₂ Tr]TpTpCE	1	266 240 (sh)	250	1.52	0.00	0.25		0.61	10.8
	2	266 233 (sh)	250	1.32	0.00	0.24		0.72	11.0
d-[(MeO) ₂ Tr]TpTpTpTpCE	1	237 (sh) 265	245	0.87	0.00	0.16		0.56	18.0
	2	237 (sh) 265	245	0.83	0.00	0.10		0.46	21.1
d-[(MeO) ₂ Tr]Tp(TpTp) ₃ TpCE	1	265	240	0.66		0.07	0.33	0.63	19.2
	2	265	238	0.59		0.01	0.22	0.40	19.6
d-[(MeO) ₂ Tr]Tp(TpTp) ₄ TO β B	1	263	238	0.69			0.38	0.68	19.9
	2	263	237	0.70			0.25	0.63	19.8
d-(TpTp),TpT	1	265	235	0.36					17.1
	2	265	234	0.33					19.1

^a Protected oligomers, measured in 95% ethanol; deprotected oligomers, measured in water, pH 7.

^b R_f values in methanol/chloroform solvent.

^c Protected oligomers, HC Pellosil; deprotected oligomers, Partisil ODS-2.

protected dimer and tetramer blocks, respectively. Finally, the dimer d-TpTO β B was added to the 3'-end of the protected octamer to give the fully protected decamer. The conditions used in these reactions and the yields obtained are given in Table I. Each fully protected oligomer was characterized by ultraviolet spectroscopy, silica gel thin layer chromatography, and silica gel high pressure liquid chromatography as described in Table II.

The protecting groups were removed sequentially by treatment with 1) hydrazine hydrate to remove the 3'-O- β -benzoylpropionyl group; 2) tetra-*n*-butylammonium fluoride (13, 14) and ammonium hydroxide in pyridine to remove the *p*-chlorophenyl phosphate protecting groups, and 3) 80% acetic acid to remove the 5'-O-dimethoxytrityl protecting group. The resulting decamers, d-(TpTp)₄TpT-1 and -2 were each purified by paper chromatography. The UV spectral properties and the chromatographic mobilities of each isomer are given in Table II.

Treatment of d-(TpTp)₄TpT with Nucleases—Snake venom phosphodiesterase slowly cleaves the phosphodiester linkages of d-(TpTp)₄TpT. The progress of these reactions was fol-

lowed by reversed phase HPLC. The pattern of products formed with time suggested that hydrolysis begins at the 3'-end of the oligomer and proceeds in a stepwise manner toward the 5'-end of the molecule. For example, early time points showed the formation of d-pTpT, d-(TpTp)₃TpT, and d-(TpTp)₂TpT, while later time points showed the disappearance of the longer oligomers with concomitant formation of increasing amounts of d-pTpT and the appearance of d-TpT. As shown in Fig. 2a, the rates of hydrolysis of the (3' \rightarrow 5') phosphodiester linkage in both isomer 1 and isomer 2 are very similar. Under the conditions of the experiments, d-(Tp)₉T, which contains exclusively phosphodiester linkages is hydrolyzed completely to monomers within 1 min.

Prolonged digestion of d-(TpTp)₄TpT by snake venom phosphodiesterase gave d-TpT and d-pTpT in ratios of 1:3.8 (isomer 1) and 1:4.0 (isomer 2). These ratios are consistent with the structure of the decamer. No cleavage of the phosphonate linkages of either isomer could be detected. In contrast to their behavior with snake venom phosphodiesterase, both isomers of d-(TpTp)₄TpT were totally resistant to hydrolysis by spleen phosphodiesterase.

As shown in Fig. 2b, the phosphodiester linkages of d-(TpTp)₄TpT-1 are cleaved more rapidly than those of isomer 2 by micrococcal nuclease although the rates of hydrolysis of both oligomers is much slower than that of d-(Tp)₉T. Cleavage appears to occur in a random manner as evidenced by the immediate production of oligonucleotides in the series d-(TpTp)_n (*n* = 1, 2, 3) and d-TpTp)_nTpT (*n* = 1, 2, 3). Initial cleavage at the 3'-terminal phosphodiester linkage to give d-TpT and d-(TpTp)₄ does not appear to occur in either isomer. No cleavage of the phosphonate linkages was observed. Under the conditions of this experiments, d-(Tp)₉T is cleaved to oligonucleotides in less than 1 min.

Neither the phosphonate nor the phosphodiester linkages of either isomer of d-(TpTp)₄TpT were hydrolyzed by S₁ nuclease even after 6 h incubation at 37°C. Under these conditions, d-(Tp)₉T was hydrolyzed to a mixture of shorter

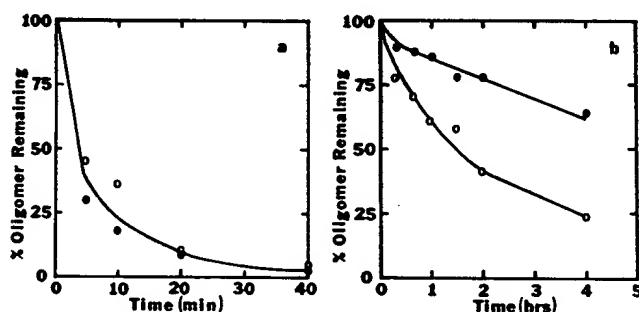


FIG. 2. Hydrolysis of d-(TpTp)₄TpT, isomer 1 (●) and isomer 2 (○) by (a) snake venom phosphodiesterase and (b) micrococcal nuclease at 37°C. Reaction conditions are described under "Materials and Methods."

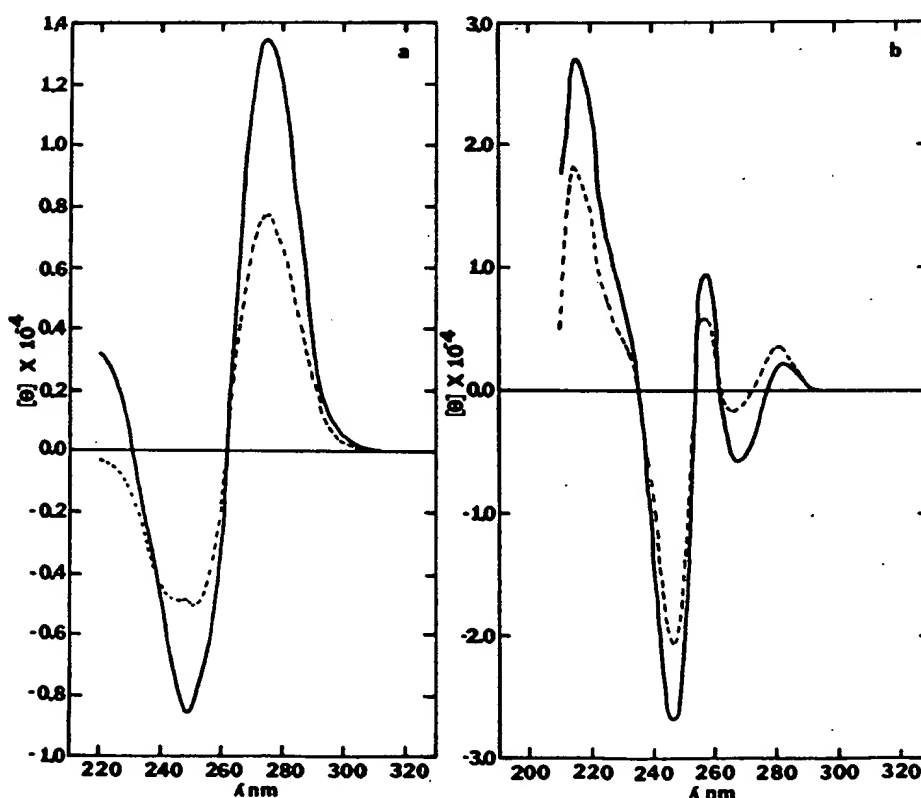


FIG. 3. Circular dichroism spectra of (a) d-(TpTp)₄TpT-isomer 1 (—) and isomer 2 (---), (b) poly(dA)·isomer 1 (—) and isomer 2 (---) in 0.50 M sodium chloride/sodium cacodylate, pH 6.8 at 4.0°C.

TABLE III
Complexes formed by oligodeoxythymidylates and complementary polynucleotides

Oligomer	Poly(dA)			Poly(rA)		
	Complex ^a	T_m^b °C	ΔH forma- tion ^c	Complex ^a	T_m^b °C	ΔH forma- tion ^c
d-(Tp) ₉ T	1T·1A	22.5	-8.9	1T·1A	18.0	-7.9
d-(TpTp),TpT	1T·1A	33.5	-10.3	1T·1A	19.5	-7.7
Isomer 1				2T·1A	17.0	-13.6
Isomer 2	2T·1A	2	-16.0	No complex		

^a The stoichiometries of the complexes were determined by continuous variation experiments (1, 5) in 0.10 M sodium cacodylate, pH 6.8 at 0.5°C, at a total nucleotide concentration of 3.5×10^{-5} M.

^b The T_m values are the transition midpoints of 1:1 or 2:1 mixtures of the oligothymidylates with poly(dA) or poly(rA). These experiments were carried out in 0.10 M sodium cacodylate, pH 6.8, at a total nucleotide concentration of 3.5×10^{-5} M.

^c kcal/mol base pair or base triplet in 0.50 M sodium chloride/sodium cacodylate, pH 6.8.

oligonucleotides within 5 min.

Physical Studies on d-(TpTp),TpT and Interaction with Polynucleotides—The CD spectra of both isomers of d-(TpTp),TpT are shown in Fig. 3a. The spectrum of isomer 1 is virtually identical both qualitatively and quantitatively with that of d-(Tp)₉T measured under the same conditions. The spectrum is qualitatively similar to that of d-(pT)₁₀ measured in 0.02 M NaCl (15) whose values of $[\theta]$ at 277 nm and 250 nm are 1.14×10^4 and -0.67×10^4 , respectively. The molecular ellipticity of the peak and trough of isomer 2 is reduced by 43% with respect to that of isomer 1 or d-(Tp)₉T.

The interactions of d-(TpTp),TpT-1 and d-(Tp)₉T with poly(dA) and poly(rA) are summarized in Table III. With poly(dA), both d-(TpTp),TpT-1 and d-(Tp)₉T form double-stranded complexes while d-(TpTp),TpT-2 forms a triple-stranded complex. With poly(rA), d-(Tp)₉T forms a double-stranded complex, d-(TpTp),TpT-1 forms both double- and triple-stranded complexes, but no complex formation is observed with d-(TpTp),TpT-2. The melting profiles of the complexes indicate that they melt in a cooperative manner.

The CD spectra of the complexes formed by d-(TpTp),TpT and poly(dA) are shown in Fig. 3b. The spectrum of poly(dA)·isomer 1 is almost identical with that of poly(dA)·d-(Tp)₉T and to that of poly(dA)·poly(dT) (16), while the spectrum of isomer 2·poly(dA)·isomer 2 shows a reduction in molecular ellipticity. Similar results were obtained for the complexes formed with poly(rA) (data not shown). The CD spectra of poly(rA)·isomer 1 and poly(rA)·d-(Tp)₉T were virtually identical, while the molecular ellipticity of the isomer 1·poly(rA)·isomer 1 triplex was reduced between 25% and 40%.

The effects of sodium ion concentration on the melting temperatures of the poly(dA)·oligo(dT) complexes are shown in Fig. 4a. Plots of T_m versus $\log [Na^+]$ are linear over the range 0.1 to 0.5 M. The stoichiometry of the complexes was constant over the range of salt concentrations studied. Similar results were obtained for the complexes formed with poly(rA) (data not shown). The slopes of the lines obtained ($\Delta T_m/\Delta \log [Na^+]$) were: poly(rA)·d-(Tp)₉T, 16.0°; isomer 1·poly(rA)·isomer 1, 11.4°; poly(rA)·isomer 1, 7.6°.

The effects of oligonucleotide concentration on the melting temperatures of the poly(dA)·oligo(dT) complexes in 0.50 M Na⁺ are shown in Fig. 4b. In these experiments, the base ratios of poly(dA) to decamer were maintained at a constant value for each complex while the total nucleotide concentration was varied out the range 8.76×10^{-6} M to 1.25×10^{-4} M. The slope of each line was determined by a least squares analysis of the data and the enthalpies of complex formation were determined from the relationship $n\Delta H = 2.303 R \log C_m/\Delta(1/T_m)$ where n is the chain length of the decamer (17, 18). Similar plots were obtained for the complexes formed with poly(rA). The

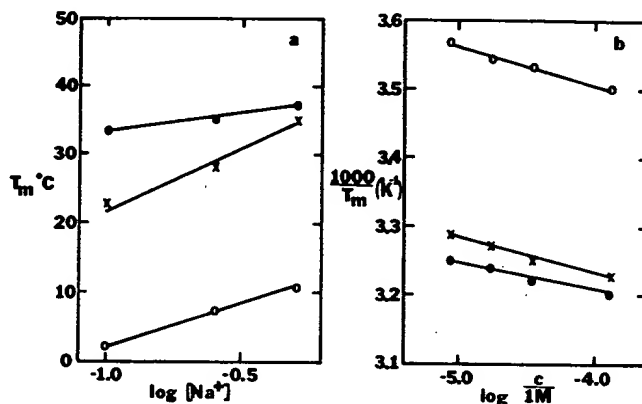


FIG. 4. Effect of (a) sodium ion concentration, (b) oligonucleotide concentration on the T_m values of poly(dA)·d-(Tp)₉T(X), poly(dA)·d-(TpTp),TpT-isomer 1 (●), d-(TpTp),TpT-isomer 2·poly(dA)·d-(TpTp),TpT-isomer 2 (○). The buffer in b was 0.50 M sodium chloride/sodium cacodylate, pH 6.8.

enthalpies of formation of these complexes are summarized in Table III.

DISCUSSION

The decathymidylate analogues were prepared by a combination of methods previously used to synthesize dideoxyribonucleoside methylphosphonates (3) and oligonucleotide phosphotriesters (19). This approach allows the preparation of analogues containing a stereoregular, alternating methylphosphonate/phosphodiester backbone. The key step in the synthesis is the preparation of the dimer, d-[(MeO)₂Tr]-TpTOβB and separation of its two diastereoisomers. The β-benzoylpropionyl group was used to protect the 3'-hydroxyl since it improves the separation of the two diastereoisomers and since it can be removed selectively under essentially neutral conditions with hydrazine hydrate (10).

Each diastereoisomer of d-[(MeO)₂Tr]TpT was phosphorylated using a modification of a method described by Agarwal and Riftina (20) to give d-[(MeO)₂Tr]TpTpCE. Dimers containing methylphosphonate linkages of the same configuration were then joined together via phosphotriester linkages to give full protected decamers, d-[(MeO)₂Tr]Tp(TpTp),TOβB.

The 3'-benzoylpropionyl groups were first removed from the protected decamers. In order to limit possible degradation of either the phosphonate or phosphodiester backbone, the decamers were then treated with tetra-n-butylammonium fluoride to remove most of the p-chlorophenyl groups (13, 14), followed by overnight treatment with ammonium hydroxide at 4°C. The dimethoxytrityl group was removed with 80% acetic acid. This procedure gave d-(TpTp),TpT as the major

product of the reaction along with traces of shorter oligomers. The latter probably arose as a result of random cleavage of the internucleotide bonds at the phosphotriester linkages by either fluoride ion or hydroxide ion (21).

Although both the phosphonate and phosphodiester linkages of $d\text{-(TpTp)}_n\text{TpT}$ are resistant to hydrolysis by spleen phosphodiesterase and by S_1 nuclease, the phosphodiester linkages of the decamers are cleaved slowly by snake venom phosphodiesterase and by micrococcal nuclease. In the case of the snake venom enzyme, analysis of the digest by HPLC suggested that hydrolysis occurs in an exonucleolytic manner starting at the 3'-phosphodiester linkage and proceeding toward the 5'-end. Similar results were obtained by Agarwal and Riftina (22) for the hydrolysis of $d\text{-(TpTp)}_n\text{TpT}$ by this enzyme. Since the enzyme is known to require a free 3'-hydroxyl group, these results suggest that the enzyme can bypass the terminal phosphonate linkage and cleave the phosphodiester bond 2 base residues removed from the 3'-hydroxy terminus. The initial rates of hydrolysis of both isomers of $d\text{-(TpTp)}_n\text{TpT}$ are not affected significantly by the configuration of the phosphonate methyl group but are considerably slower than that of $d\text{-(Tp)}_n\text{T}$. This difference in rate may be due to the reduction of the negative charges which could reduce the affinity of the enzyme for the analogue, as well as to the presence of nonionic methylphosphonate groups adjacent to the susceptible phosphodiester linkage.

Under the conditions of our experiments, we found no cleavage of the phosphonate linkages in either isomer of $d\text{-(TpTp)}_n\text{TpT}$, even after prolonged (24 h) incubation. The final products of the reaction, $d\text{-(Tp)}_n\text{T}$ and $d\text{-(pTp)}_n\text{T}$, were obtained in the expected ratios. These results are in contrast to those of Agarwal and Riftina who reported that the phosphonate linkages of $d\text{-(Tp)}_n\text{T}$ are hydrolyzed slowly in the presence of large amounts of snake venom phosphodiesterase (22).

The internucleotide phosphodiester linkages of $d\text{-(TpTp)}_n\text{TpT}$ are cleaved slowly in a random manner by micrococcal nuclease. The initial hydrolysis of isomer 1 appears to occur more slowly than that of isomer 2, although both isomers are cleaved much more slowly than is $d\text{-(Tp)}_n\text{T}$. Recent evidence suggests that the binding of micrococcal nuclease to oligo- and polynucleotides involves ionic or hydrogen-bonding interactions between the enzyme and up to three adjacent phosphodiester groups of the oligonucleotide (23-25). Since each phosphodiester linkage in $d\text{-(TpTp)}_n\text{TpT}$ is flanked by a nonionic methylphosphonate linkage, the ability of the enzyme to bind correctly to these analogues could be considerably diminished. The difference in the rates of hydrolysis of the two isomers suggest that the configuration of the methylphosphonate group may influence this binding process.

The conformation of $d\text{-(TpTp)}_n\text{TpT}$ was studied by CD spectroscopy. The near identity of the CD spectra of isomer 1 and of $d\text{-(Tp)}_n\text{T}$ suggests that both oligomers have very similar conformations. On the other hand, isomer 2 shows a reduction in molecular ellipticity. Similar reductions in the $[\theta]$ of $d\text{-(pTp)}_n$ have been observed in the presence of high concentrations of sodium perchlorate (15). The cause of this decrease was ascribed to the lower activity of water with a concomitant change in the solvation and conformation of the oligomer. Since the $[\theta]$ of isomer 2 is less than those of isomer 1 and $d\text{-(Tp)}_n\text{T}$, this reduction most likely reflects a difference in the conformation of isomer 2 compared to that of isomer 1.

Recent proton magnetic resonance investigations of the sugar-phosphonate backbones of a series of dideoxyribonucleoside methylphosphonates including $d\text{-(TpT)}$ (4), have shown that the sugar puckering and rotamer populations about ψ , ϕ , and ϕ' are very similar in both diastereoisomers.

Examination of molecular models does not reveal any obvious differences between steric restrictions to rotation about the bonds of the methylphosphonate linkages in these diastereoisomers. Thus, the apparent differences in the conformation between isomer 1 and isomer 2 could result from differences in solvation of the two isomers. Such differences could lead to changes in the N -glycosyl torsion angle as well as to changes in the weak intramolecular base-base interactions.

A similar suggestion was made to account for the effects of methylphosphonate configuration on the base-stacking interactions in the dimer $d\text{-ApA}$ (1, 4). In this case, the dimer with the S configuration has a base-stacking pattern similar to that of $d\text{-ApA}$, while the dimer with the R configuration has reduced base-stacking interactions with the bases oriented in a parallel manner. In the stacked conformation of $d\text{-ApA}$, the methyl group with the S configuration lies near the hydrophobic base-stacking region, while the methyl group with the R configuration points away from this region toward the solvent.

Both isomers of $d\text{-(TpTp)}_n\text{TpT}$ form complexes with poly(dA). The stoichiometry and CD spectrum of the poly(dA)·isomer 1 duplex is identical with that of the poly(dA)· $d\text{-(Tp)}_n\text{T}$ duplex, while isomer 2 forms a 2T·1A triplex whose CD is reduced relative to that of poly(dA)·isomer 1. At low sodium ion concentration, the T_m of poly(dA)·isomer 1 is 11°C higher than that of poly(dA)· $d\text{-(Tp)}_n\text{T}$. Since the structures of these two duplexes appear to be very similar, the increased thermal stability of poly(dA)·isomer 1 may be attributed to the decrease in charge repulsion between the partially nonionic backbone of $d\text{-(TpTp)}_n\text{TpT}$ -1 (four negative charges per molecule of decamer) and phosphate groups of poly(dA) as compared to the charge repulsion between the backbone of $d\text{-(Tp)}_n\text{T}$ (nine negative charges per molecule of decamer) and poly(dA). Similar effects have been observed previously for the interactions of deoxyribooligonucleotide alkyl phosphotriesters with complementary polynucleotides (1, 5, 6).

The effect of salt concentration on the T_m of the complexes agrees with this assessment. Thus, the T_m of poly(dA)· $d\text{-(Tp)}_n\text{T}$ increases more rapidly with increasing sodium ion concentration than does the T_m of poly(dA)·isomer 1. The effect of sodium ion concentration on the T_m of the triple-stranded complex, isomer 2·poly(dA)·isomer 2, is intermediate between that of poly(dA)·isomer 1 and poly(dA)· $d\text{-(Tp)}_n\text{T}$. The net number of negative charges in the backbone of the two $d\text{-(TpTp)}_n\text{TpT}$ oligomers of isomer 2·poly(dA)·isomer 2 is eight, which is one less than that of $d\text{-(Tp)}_n\text{T}$ in the poly(dA)· $d\text{-(Tp)}_n\text{T}$ duplex. This slight reduction in charge density coupled with the difference in the structures of the two complexes could account for the slightly lower dependence of the T_m of the triple-stranded complex on salt concentration.

The difference in stability of poly(dA)·isomer 1 versus poly(dA)· $d\text{-(Tp)}_n\text{T}$ is reflected in the enthalpy of formation of the two duplexes. In 0.5 M sodium chloride/sodium cacodylate buffer where the melting temperatures of the duplexes are approximately equal, the ΔH of poly(dA)· $d\text{-(Tp)}_n\text{T}$ is -8.9 kcal/mol base pair while that of poly(dA)·isomer 1 is -10.3 kcal/mol base pair. The difference in ΔH , 1.4 kcal/mol base pair, may in part be due to the change in enthalpy resulting from partial replacement of the phosphodiester linkages of the decamer with nonionic methylphosphonate linkages. A similar difference, 1.6 kcal/mol base pair, was observed for the enthalpy of formation of poly(dA)· $d\text{-(Tp)}_n\text{T}$ versus poly(dA)· $d\text{-(Tp(Et))}_n\text{T}$ (5).

The interactions of $d\text{-(TpTp)}_n\text{TpT}$ with poly(rA) are different than those observed with poly(dA). Isomer 1 forms both

double- and triple-stranded complexes with poly(rA), while no complex is observed for isomer 2. Based on the similarity of their CD spectra, the structures of poly(rA)·isomer 1 and poly(rA)·d(Tp)₉T appear to be identical.

The T_m values of poly(rA)·isomer 1 and poly(rA)·d(Tp)₉T are lower than those of the corresponding complexes with poly(dA). A similar effect was observed for poly(rA)·poly(dT) whose T_m is 4.4°C lower than that of poly(dA)·poly(dT) in 0.1 M sodium citrate buffer (26). T_m values of poly(rA)·isomer 1 and poly(rA)·d(Tp)₉T are quite similar in 0.1 M buffer in contrast to the difference in values observed for the poly(dA) complexes. The effect of salt concentration on thermal stability follows the same order as observed for the poly(dA) complexes; the greatest increase is observed for poly(rA)·d(Tp)₉T, followed in order by isomer 1·poly(rA)·isomer 1 and poly(rA)·isomer 1. Again, this order parallels the charge density in oligomer backbones of the complexes. However, the similarity of the T_m and ΔH values of poly(rA)·d(Tp)₉T and poly(rA)·isomer 1 suggests that any stability gained by reduction of charge repulsion is offset by other factors which tend to destabilize the poly(rA)·isomer 1 complex.

The complexes formed by the two isomers of d-(TpTp)₉TpT with complementary polynucleotides have different stoichiometries and thermal stabilities. X-ray crystallographic studies (27) and CD investigations (28) have shown that the duplex poly(dA)·poly(dT) exists in a B-type geometry while the triple-stranded complex, poly(dT)·poly(dA)·poly(dT) assumes an A-type geometry. The identity between the stoichiometries and the CD spectra of poly(dA)·poly(dT), poly(dA)·d(Tp)₉T, and poly(dA)·isomer 1 duplexes suggests that the latter assumes a B-type geometry in solution. In analogy with poly(dT)·poly(dA)·poly(dT), the triple strand complex isomer 2·poly(dA)·isomer 2 has an A-type geometry. In the case of the complexes formed between the decamers and poly(rA), an A-type of geometry would be expected (29–31).

Although the absolute configurations of the methylphosphonate groups of isomers 1 and 2 have yet to be determined, we might speculate on possible reasons for the differences in complex formation by these two oligomers. Examination of Kendrew molecular models of nucleic acid duplexes in both the A- and B-type geometry does not reveal any unfavorable steric interactions which would restrict the binding of either isomer of d(TpTp)₉TpT. This is in contrast to the case of the oligothymidylate triester, d-[Tp(Et)]₉T where formation of A-type complexes appears to be unfavorable due to steric interactions involving the phosphate ethyl groups (5). The formation of a complex with either the A- or B-type geometry by d-(TpTp)₉TpT whose methylphosphonate groups have the *S* configuration would place the phosphonate methyl groups near the hydrophobic base-stacking region of the complex. In this orientation, the methyl group should have the least perturbational effect on solvent interactions with the complex. In contrast, complex formation of d-(TpTp)₉TpT whose methylphosphonate groups have the *R* configuration would orient the methyl group away from the base-stacking region and toward the solvent. Such orientation should result in unfavorable interactions between the exposed methyl groups and the surrounding solvent. Thus, the differences in stability between the complexes formed by isomer 1 and isomer 2 could arise from differences between the solvation of the oligomer backbones in the single-stranded and duplex states.

The results presented above demonstrate that introduction

of methylphosphonate groups into the backbones of oligodeoxyribonucleotides can have important effects on the ability of the oligomers to serve as substrates for nuclease enzymes and to interact with complementary polynucleotides. The unique properties of these oligonucleotide analogues suggest that they may be useful for probing the mechanisms of nucleic acid enzymes such as DNA polymerase and DNA ligase. Studies directed along these lines will be the subject of future communications.

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OFFICE OF PETITIONS

In re Application of
Brakel, et al.
Application No. 08/479,999
Filed: June 28, 1994
Title: MODIFIED NUCLEOTIDE
COMPOUNDS

DECISION ON PETITION

This is a decision on the renewed petition to revive the above-identified application under 37 CFR 1.137(b), filed December 15, 2003.

The petition is **GRANTED**.

The above-identified application became abandoned for failure to timely file a proper response to the final Office action mailed September 26, 2000, which set a shortened statutory period for reply of three months. No extensions of time under the provisions of 37 CFR 1.136(a) were obtained. Accordingly, the above-identified application became abandoned on December 27, 2000. A Notice of Abandonment was mailed on July 16, 2001. Petitioner filed a petition to revive under 37 CFR 1.137(b) on December 26, 2001. However, this petition was dismissed in a decision mailed on October 14, 2003.

On renewed petition, petitioner has drawn the Office's attention to a Notice of Appeal that was filed on December 26, 2001. In addition, petitioner has met the other requirements for a grantable petition under 37 CFR 1.137(b).

As the fee for the Notice of Appeal was not charged upon filing of the December 26, 2001 petition, the fee at the time of filing of \$320 has been charged to Deposit Account No. 05-1135, as authorized.

Please be advised that the two-month period for filing an appeal brief in triplicate (accompanied by the fee required by 37 CFR 1.17(c)) runs from the date of this decision.

The application file is being forwarded to Technology Center 1600 to await for applicant's filing of the Appeal Brief.

Telephone inquiries concerning this decision should be directed to the undersigned at (703) 305-0272.

Cliff Congo

Cliff Congo
Petitions Attorney
Office of Petitions